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# BIOLOGICAL BULLETIN

## THE LIFE HISTORY OF DESMOGNATHUS FUSCA.

*(Continued from March Issue.)*

INEZ WHIPPLE WILDER.

### The Aquatic Larval Period.

Following the brief but very important terrestrial larval period, the aquatic larval period begins. As may be computed from the limits of dates during which egg-laying occurs (June 1 to Sept. 1), the probable duration of incubation (5 weeks), and the somewhat variable period of terrestrial life (1 to 2 weeks), the date of the beginning of the aquatic period can hardly be earlier than the middle of July or later than the middle of October. I have personally never known of aquatic larvæ being found between June 17 and September 1. By September 15 they are present in the brooks in great abundance, the majority of them with yolk still present in the intestinal walls, a condition not unlike that shown by stage *F* of the terrestrial larvæ and never as yet found by me in specimens collected in October. These facts point to early September as the time when the majority of the larvæ reach the water and begin their aquatic existence. The wide range of variation in the size and proportions of larvæ during September and October (cf. Table II. and Graphs III. and IV.) represents the inevitable difference in age resulting from the long period of egg laying.

As to the duration of the larval period, we have the statement of Reed and Wright ('09): "The larvæ transform from September to December, when they are from 18 to 20 mm. long." My own observation does not confirm this statement. It is a very significant fact that after the larvæ once appear in the water in September, they are found about equally abundantly

during the fall, winter, and early spring months, and that, as shown by Table II. and its accompanying Graphs III. and IV., the average proportions and size of the body remain practically the same until late spring, when there occurs a marked increase in average size and a decided change in the proportionate lengths of the regions of the body in that the tail lengthens more rapidly than either the head or trunk. Structurally, also, the specimens taken during the fall and winter display none of the indications of approaching metamorphosis which are so unmistakable when they do appear in the May specimens. Moreover I have never found larvæ actually undergoing metamorphosis except in June, nor any of the very small recently metamorphosed adults except in June and the early part of July. The aquatic larval period would thus appear to extend practically through autumn, winter,

TABLE II.

AQUATIC LARVAL PERIOD.

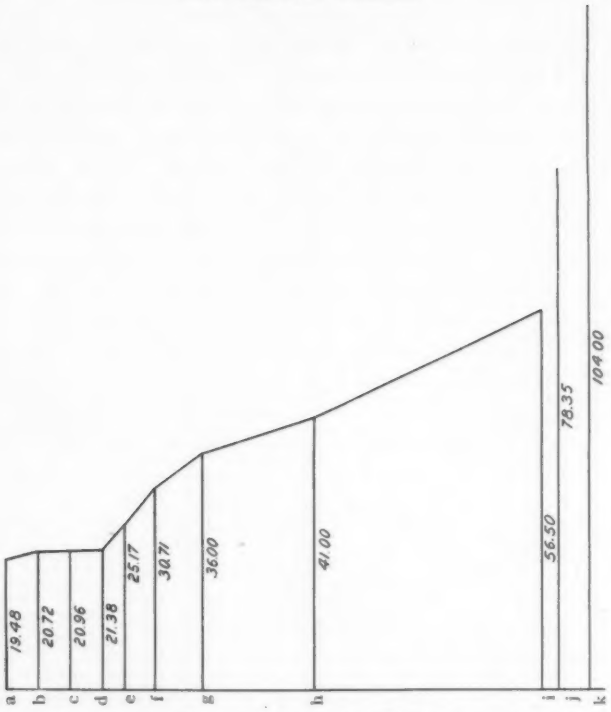
| When Collected. | Number of Examples. | Column I. Actual Sizes (Cf. Graph III.), Mm. | | | Column II. Proportionate Lengths of Body Regions (Cf. Graph IV.). | | |
|---|---|---|---|---|---|---|---|
| | | Maximum. | Minimum. | Average. | Maximum. | Minimum. | Average. |
| September and October. | 42 | Head 5.00 | 4.00 | 4.34 | .208 | .261 | .223 |
| | | Trunk 7.25 | 4.30 | 5.86 | .302 | .281 | .301 |
| | | Tail 11.75 | 7.00 | 9.28 | .489 | .458 | .476 |
| | | Total.....24.00 | 15.30 | 19.48 | 1.000 | 1.000 | 1.000 |
| November and December. | 15 | Head 5.40 | 4.00 | 4.56 | .212 | .234 | .220 |
| | | Trunk 7.60 | 5.50 | 6.38 | .298 | .324 | .307 |
| | | Tail 12.50 | 7.50 | 9.62 | .490 | .441 | .473 |
| | | Total.....25.50 | 17.00 | 20.72 | 1.000 | 1.000 | 1.000 |
| January and February. | 5 | Head 4.55 | 4.25 | 4.51 | .207 | .224 | .215 |
| | | Trunk 6.83 | 5.75 | 6.45 | .311 | .303 | .308 |
| | | Tail 10.62 | 9.00 | 10.00 | .483 | .474 | .478 |
| | | Total.....22.00 | 19.00 | 20.96 | 1.000 | 1.000 | 1.000 |
| March and April. | 8 | Head 4.50 | 4.10 | 4.395 | .191 | .217 | .206 |
| | | Trunk 7.50 | 5.20 | 6.655 | .319 | .298 | .311 |
| | | Tail 11.50 | 8.80 | 10.335 | .489 | .485 | .483 |
| | | Total.....23.50 | 18.10 | 21.385 | 1.000 | 1.000 | 1.000 |
| May. | 5 | Head 6.00 | 4.75 | 5.12 | .182 | .218 | .203 |
| | | Trunk 10.00 | 6.75 | 7.55 | .303 | .310 | .300 |
| | | Tail 17.00 | 10.25 | 12.50 | .515 | .471 | .497 |
| | | Total.....33.00 | 21.75 | 25.17 | 1.000 | 1.000 | 1.000 |

ADULT PERIOD.

| When Collected. | Number of Examples. | Column I. Actual Sizes (Cf. Graph III.), Mm. | | | Column II. Proportionate Lengths of Body Regions (Cf. Graph IV.). | | |
|---|---|---|---|---|---|---|---|
| | | Maximum. | Minimum. | Average. | Maximum. | Minimum. | Average. |
| Metamorphic stage. Collected June 17 to July 1. | 3 | Head 5.60 | 5.50 | 5.53 | .193 | .198 | .195 |
| | | Trunk 9.40 | 7.75 | 8.72 | .324 | .279 | .306 |
| | | Tail 14.00 | 14.50 | 14.17 | .493 | .522 | .501 |
| | | Total.....29.00 | 27.75 | 28.42 | 1.000 | 1.000 | 1.000 |
| Small adults. Collected June 5. | 2 | Head 6.70 | 6.50 | 6.60 | .191 | .195 | .193 |
| | | Trunk 10.50 | 10.30 | 10.40 | .300 | .309 | .305 |
| | | Tail 17.80 | 16.50 | 17.15 | .509 | .496 | .502 |
| | | Total.....35.00 | 33.30 | 34.15 Average of metamorphic and smallest adults 30.71 | 1.000 | 1.000 | 1.000 |
| Small adult. Collected Sept. 17. | 1 | Head..... | ..... | 6.50 | ..... | ..... | .181 – |
| | | Trunk..... | ..... | 10.50 | ..... | ..... | .292 – |
| | | Tail..... | ..... | 19.00 | ..... | ..... | .528 – |
| | | Total..... | ..... | 36.00 | ..... | ..... | 1.000 |
| Small adult. Collected May 3. | 1 | Head..... | ..... | 7.00 | ..... | ..... | .171 |
| | | Trunk..... | ..... | 12.50 | ..... | ..... | .305 |
| | | Tail..... | ..... | 21.50 | ..... | ..... | .524 |
| | | Total..... | ..... | 41.00 | ..... | ..... | 1.000 |
| Miscellaneous set of adults. Collected in June and July. | 10 | Head 16.00 | 9.00 | 12.35 | .154 | .159 | .158 |
| | | Trunk 31.00 | 16.50 | 23.65 | .298 | .292 | .302 |
| | | Tail 57.00 | 31.00 | 42.35 | .548 | .549 | .540 |
| | | Total.....104.00 | 56.50 | 78.35 | 1.000 | 1.000 | 1.000 |

It will be noted that in the above table the number of examples collected during September and October is greatly in excess of the number collected during the other months of the aquatic larval period. This fact is not due, as would seem to be indicated, to a greater abundance of the material during these months, but rather to the fact that these happen to be the months when most of my collection of *Desmognathus* material for laboratory purposes is made.

and spring, and to cover a somewhat variable period of from 8 to 10 months. The perennially running brooks in which they live are of course spring fed and do not freeze solid even though they are very shallow. In fact the brook which I have particularly studied as a typical *Desmognathus* habitat is said to never




GRAPH III., showing the growth in length during the aquatic larval period (lines *a–f*), metamorphosis (line *f*), and the first two years of adult life (lines *f–i*); also, for comparison with these, the average length (line *j*) and the maximum length (line *k*) of ten miscellaneous adults. Based upon the statistics given in the first column of Table II.

The time ratios are indicated by measurements horizontally. The vertical lines show, natural size, the average lengths of specimens as follows:

Aquatic larval period:

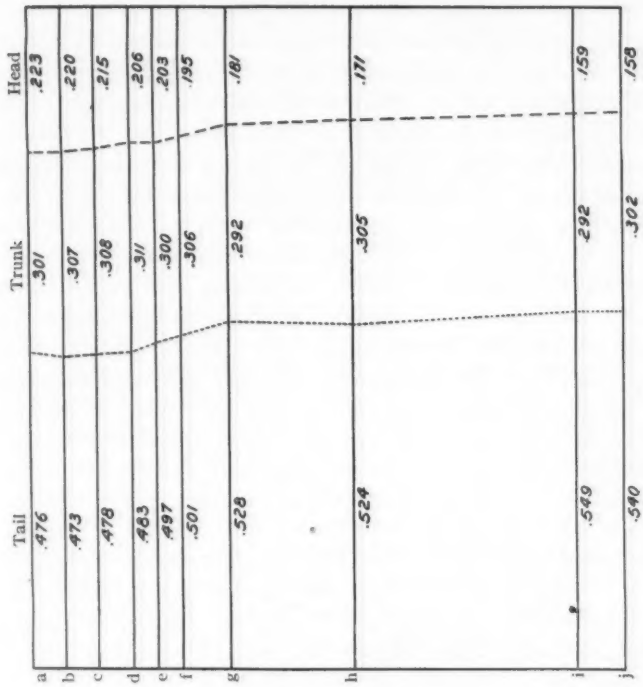
*a*, collected in September and October.
*b*, collected in November and December.
*c*, collected in January and February.
*d*, collected in March and April.
*e*, collected in May.

Metamorphosis:

*f*, collected in June and July.

Adult period:

*g*, collected in the September following metamorphosis.
*h*, collected in May nearly one year after metamorphosis.
*i*, collected in June and July two years after metamorphosis.
*j*, average of 10 miscellaneous adults.
*k*, maximum of 10 miscellaneous adults.




GRAPH IV., showing a comparison of the proportionate lengths of body regions during the aquatic larval period (lines *a–f*), metamorphosis (line *f*), and the first two years of adult life (lines *f–i*); also the proportionate lengths of body regions of the average of 10 miscellaneous adults (line *j*). Based upon the statistics given in the second column of Table II.

The time ratios shown by the horizontal measurements and the dates expressed by the location of the vertical lines are as in Graph III. Note that by the end of the second year of adult life (line *i*) the body proportions are those of the average adult (line *j*).

entirely freeze over. Thus I have always found, even in mid-winter, a little open space, sometimes only a few inches in width, and by breaking away the ice bordering the edges of this, have been able to reach the quiet pools under the ice, in which larvæ are found. Specimens have in this way been collected in January when the temperature was at 0° F. and had been at that point or lower for three or four days.

The larvæ frequent the quieter regions, always out of the current except when a temporary disturbance drives them into it. They seek not only quiet but very shallow water, where they lie motionless among the decaying leaves, which, owing to the wooded nature of their habitat, fill such pools. Such leaves also furnish quiet lurking places when they lie in a thick mass covering the surface of the more rapidly moving regions of the stream. Among the loose debris and sediment which these surroundings afford, the larvæ lie, their mottled brown color forming a perfect protective resemblance. The collector may thus at first stare several minutes into such a little pool before he recognizes in an apparent fragment of the midrib of a decaying leaf, the mid-dorsal ridge of a *Desmognathus* larva, and he may possibly realize the true nature of the object under observation only when upon disturbing the water, active swimming movements betray the living animal. On the other hand having once begun to recognize the larvæ one is frequently deceived into picking up some lifeless fragment which the larvæ so closely resemble. Several larvæ are frequently found in close proximity in the same little pool or mass of leaves, a fact which probably results from the abundance of the larvæ, but may in the early fall be due to the simultaneous arrival, in the same little pool, of several individuals of the same brood.

Frequently the larvæ, when concealing themselves among the leaves, lie half out of the water, the body immersed just sufficiently to bring the gills under the surface. This habit I have observed particularly in the case of individuals in captivity, and have thought that it might be associated with lack of sufficient oxygen. However the larvæ are certainly not adapted to the pressure conditions of deep water, and although they are quiet and apparently comfortable when in captivity in water a centimeter deep, if the depth of the water be gradually increased they show signs of uneasiness when it becomes about two centimeters, and by the time a depth of three or more centimeters is reached they make frantic efforts to reach the top of the water, where, however, they can sustain themselves only by active swimming or by resting upon some surface. In fact under these conditions they almost invariably swim to the edge of the aquarium, run

rapidly up its side, and attempt to make their escape, while in shallow water they remain for days in an open dish and make no such attempt. This adjustment to shallow water is undoubtedly a means for insuring that they remain in regions where at the time of metamorphosis they can reach the necessary terrestrial environment; for the adults, being lungless, have no hydrostatic organs, neither have they any method of aquatic respiration other than the skin.

The larvæ react vigorously to attempts to capture them by any seizing act or any method which involves pressure against the opposite sides of the body, being evidently organized to escape from jaws and teeth. That they frequently barely succeed in making such escapes is evidenced by the large proportion of maimed individuals which one finds, tails and hind legs being the parts most frequently lost. The location of the lateral line organs or neuromasts upon opposite sides along the lateral surfaces of the body may have some significance in connection with this reaction. On the other hand, the larvæ do not appear to be especially sensitive to mere tactile stimuli even when these are applied to the regions where the neuromasts are most abundant, unless such stimuli are applied upon two opposing regions. After the application of such opposing stimuli, however, or the repeated application of single stimuli, the sensitiveness of the larva to touch seems to be for a time considerably augmented, and a stimulus applied to a single region will then give rise to vigorous efforts to escape.

On the other hand the larvæ appear to have no reflex mechanism for protection against forces which gently raise and lower the body, like the slight natural movements of quiet water. They may therefore be readily captured by lifting them with the hand, a watch glass, or any other concave object which may be passed gently under them and then quietly raised; the gradual flowing of the water from the surface upon which they are thus lifted produces also no disturbance so long as the larva remains moist. Larvæ may in this way be transferred from their natural habitat to the collecting jar, without a single movement on their part, while the slightest pinching or seizing force results almost invariably in such violent wriggling that the animal makes its escape.

The food of larvæ during the aquatic period consists mainly of little copepods and occasionaly very small aquatic insect larvæ. Mingled with the debris of such food in the stomach and intestine, there are usually found considerable quantities of sand and disintegrating vegetable matter, the ingestion of which is incident to the capture of the living food. The mouth opening does not extend very far back and it is equipped at each angle with a labial fold (*lf*, Fig. 17) so arranged that when the lower jaw is depressed the folds are stretched across the angles making the orifice almost circular in form and directed slightly downward. This is evidently an adaptation to the capture of such food forms as may be scooped up or sucked in from among the sediment at the bottom or upon the surfaces of decaying leaves.

The respiration during the larval period is accomplished by means of the three pairs of external gill bushes. Although these consist of relatively few filaments, they are held so widely outspread in the water as to render their position the most advantageous one possible to the performance of their function. When the larva is disturbed, however, as by opposing pressure applied to the lateral surfaces of the head, the gills are quickly drawn back and held closely appressed against the sides of the body, a protective reflex the efficiency of which is shown by the fact that one never finds larvæ with the gills injured.

With four gill slits upon each side, it would seem that the mechanism must be present for producing a flow of water through the mouth and pharynx and out over the gills, yet I have never been able to detect the slightest evidence of such a current, or of the rhythmic movements of the floor of the mouth and pharynx such as would be necessary to produce it. Occasionally there may be observed, however, a single, vigorous little movement of the gill bushes, which is undoubtedly for the purpose of assisting diffusion by hastening the change of the water in contact with the gills. This movement is usually performed once or twice when the larva comes to rest after vigorous swimming. The usual position of the larvæ when at rest, upon or among decaying leaves just below the surface of the water, gives natural access to the region of the water which is most completely aerated.

The gill filaments present a certain glistening white appearance

which at times renders them the most conspicuous portions of the body. This appearance is similar to certain white spots which occur along the lateral surfaces of the body and occasionally in other regions, in both the larval and the adult stages, and have been described as "white pigment spots." There is considerable evidence, however, that in the gills, at least, the phenomenon is due to an accumulation of some gas, presumably carbon dioxide, in the tissues, but its exact nature and significance demand further investigation.

In the matter of internal structure as in size, the aquatic larva appears to be in a general static condition throughout the larger part of its larval life. Late in the spring, however, shortly before the metamorphosis, a series of changes becomes inaugurated, involving especially the integument and the organs derived from it, as well as certain skeletal parts, and leading rapidly to the conditions which characterize the adult. Such changes are to be considered as belonging particularly to the period of metamorphosis, and will be described as such.

Until such distinctive metamorphic changes appear, the skin of the larva remains in practically the condition attained at the end of the terrestrial period. The two layers of cells of the epidermis remain fairly well defined. The cuticular border of the outer layer becomes thicker and more compact in appearance, and the cap of intracellular pigment between this border and the nucleus becomes denser. In the deeper layer, the cells of which rest directly upon the dense corium, the distended vacuolated Leydig cells are in most regions so abundant that each is usually separated from the neighboring Leydig cells by but a single circle of cells of the general epidermal type (Plate IV., 25). Here and there among the cells of the deeper layer are cells which are considerably elongated and somewhat piriform and which extend out between the cells of the outer layer almost to the external surface. These are the cells which in the German literature are designated as the "Schaltzelle" (Plate IV., 23, *intr*). As the larval period advances there is a gradual increase in the thickness of the epidermis due to the increasing size of the cells of the deeper layer, especially the Leydig cells, which gradually become more distended, while their nuclei become notice-

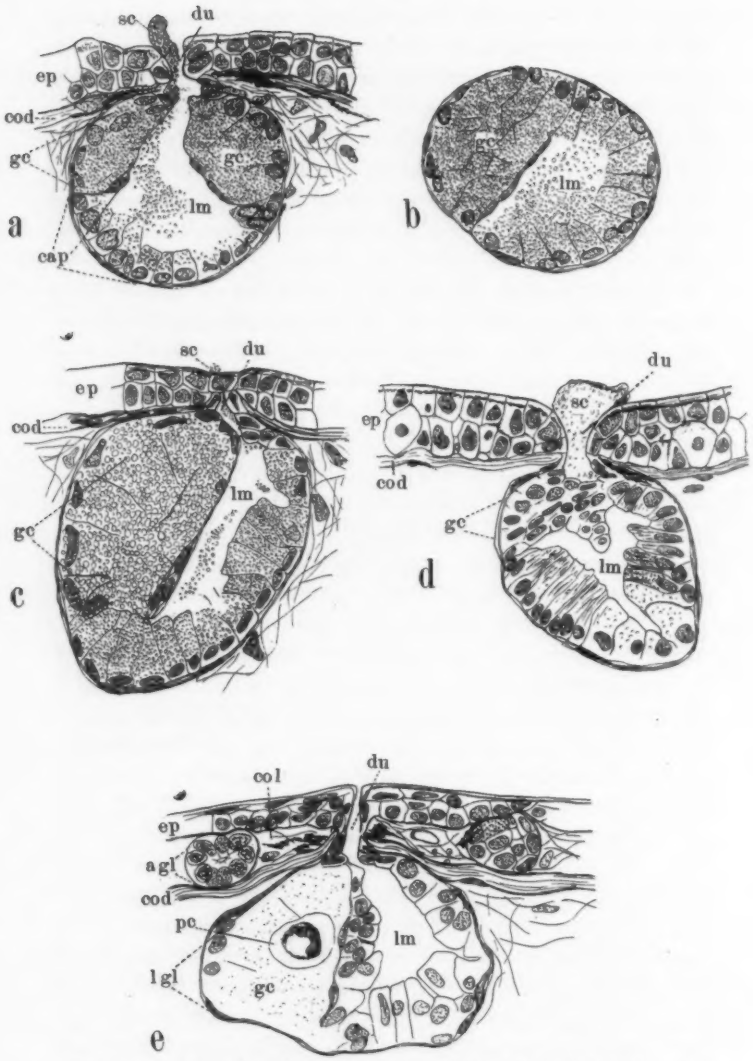



FIG. 15. Sections through larval acinous glands; (*a*) vertical section, *i. e.*, at right angles to the external surface, through the middle of a gland of the suprabranchial group of a recently hatched terrestrial larva; (*b*) cross section, *i. e.*, parallel with the external surface, through the middle of one of the mid-dorsal

ably smaller. The nuclei of the cells of both layers appear in many cases irregular in form and often show sharp constrictions which suggest amitotic division, a suspicion further confirmed by the increasing number of multinucleate cells which, in spite of the absence of mitotic phenomena, appear in the outer layer of the epidermis, particularly in the latter part of the larval period. As the Leydig cells become larger with the continuance of larval life, some of the surrounding cells of the deeper layer become crowded together into a columnar form, while others appear to be pushed out of line, some lying in the deeper region of the epidermis and others becoming crowded into the angles between the bases of the cells of the outer layer (Plate V., 29). Thus the number of cells which reach from the deeper layer into the outer layer becomes increased. These, however, seem not to actually belong to the external layer since, even if they reach the surface, they apparently do not acquire a cuticular border. These slight changes in the epidermis are accompanied by a gradual increase in the thickness of the dense corium, from which, moreover, connective tissue cells gradually invade the epidermis. These together with leukocytes and branches of nerve fibers may thus be seen among the cells of the deeper layer, and not infrequently these invaders, as well as the pigment cells, actually break through the thin membrane of the Leydig cells, and lie within the large vacuolated spaces which these afford.

The acinous glands (Fig. 15), which have already developed and begun to function at the time of hatching, remain in a fully

group of glands of a terrestrial larva; (*c*) vertical section through one of the glands of the supra-branchial group of an aquatic larva in late spring, hence near the end of the larval period; (*d*) vertical section through one of the supra-branchial group of an aquatic larva in February, showing an almost completely discharged condition induced by prolonged mechanical stimulation immediately before the specimen was killed; (*e*) vertical section through one of the supra-branchial group of an individual immediately after metamorphosis, showing the gland in an empty condition and undergoing atrophy, while in the loose corium nearby are seen two of the newly developed acinous glands of the adult period of life. Note the position of the larval gland below the level of the dense corium, while the adult glands are in their permanent location external to this layer.

*Agl*, adult gland; *cap*, capsule of the gland; *cod*, dense corium; *col*, loose corium; *du*, duct of the gland; *ep*, epidermis; *gc*, giant cells of the gland; *lm*, lumen of the gland; *pc*, pigment cell which has invaded the gland; *sc*, secretion discharged from the gland. Drawn with Abbé camera. × 225.

active condition throughout larval life. These glands, arranged as above described (cf. p. 291 Vol. XXIV. *Biol. Bull.*), lie quite below the dense corium, embedded in the subcutaneous connective tissue, and communicating with the external surface by means of a narrow duct. Although of the type of amphibian gland variously designated as "granular" or "poison," they do not in detail correspond in all respects to the descriptions of such glands in other amphibians. They vary greatly in size, the largest when in a distended condition having a diameter eight times the thickness of the skin, while the smallest when distended have a diameter only twice the thickness of the skin. They are spherical or subspherical in external form; the larger ones, especially those of the suprabranchial group, are not of the simple acinous type which this external form suggests, however, since internally the lumen is partially subdivided by invaginations of the single layer of gland cells, as well as by the occurrence here and there of groups of these cells which are much taller than the rest and thus encroach upon the lumen. The connective tissue sheath (*cap*) of the gland follows closely the invaginations. The glands of all sizes are particularly characterized by the development of certain cells into the "giant" type (*gc*) through an enormous accumulation of granules of the same sort apparently as those which fill the other cells of the gland. These giant cells are differentiated very early in the development of the glands, even while mitosis is still in progress and before the lumen of the gland has appeared; in all of the glands they comprise at least one group of some eight or ten cells which lie near the orifice of the gland upon one side and become almost completely surrounded and cut off from the rest of the gland by the ingrowth of the connective tissue sheath (Fig. 15, *b*). Thus placed they form a mass nearly equal in volume to half of the whole gland, so that a section through the middle of the gland parallel with the external surface of the skin often shows the remainder of the gland wrapped in a crescentic form about this mass of giant cells. Giant cells occur also in other parts of the larger glands, either as isolated cells or in small groups of two or three. The granules filling all of the gland cells are similar, except that those in the giant cells become much larger. The ordinary cells, however, appear to constantly dis-

charge their contents into the lumen of the gland, where they form into a fluid which may often be seen in the sections in the process of being discharged through the gland duct. The discharge of the granular contents of the gland cells seems to involve a rupture of the cell membrane. The giant cells, on the other hand, seldom appear to be discharging their granules even when the remainder of the gland shows great activity, although the membranes of a group of such cells not infrequently appear to be ruptured, and a large accumulation of granules to be ready to be discharged. When, however, a larva is killed after having been subjected to much mechanical stimulation in the nature of poking and seizing with the forceps, not only are the ordinary gland cells found empty or nearly so, but the giant cells also are empty and the mass of cells, separated from the rest of the gland by its sheath, is collapsed (Fig. 15, *d*). It thus seems that these giant cells are in the nature of a reserve supply, ready for instant response in an extreme emergency when the constant secretive action of the ordinary gland cells is inadequate. The only other possible explanation is that the secretions of the ordinary and the giant cells are different in function, a supposition which may upon further investigation prove to be correct, although the appearance of the secretions within the cells gives no indication of such a difference.

The condition of the nuclei of the gland cells in the fully discharged condition does not in the least indicate that the cells are doomed to degeneration; the nuclei are large and round and appear fully vigorous, much like the nuclei of the epidermal cells. Neither does it appear that there is any provision for the replacement of glands during the larval life, either in the form of single replacement cells or the anlagen of new glands; and, as all the glands, both large and small, are equally mature and active and exist in practically the same numbers throughout the whole larval life, there is no chance for the supposition that the small ones grow into the large ones. We are forced, then, to the belief that the larva hatches fully equipped with a set of glands of the granular type that are to perform their function without renewal throughout the many months of larval life.

That this function is important is very evident, though its

exact nature is a problem. The usual explanation of the function of the granular type of gland in the Amphibia is that its secretion is of a poisonous nature and protects the animal from capture by other forms which might use it as food. The full discharge of the contents of the glands by the *Desmognathus* larva under prolonged mechanical stimulation seems to corroborate this explanation, as does also the special supply of these glands in the region of those extremely important organs, the gills. On the other hand, the enormous development which this type of gland reaches in forms like *Bufo* and *Plethodon*, which have become very terrestrial, seems to indicate a correlation, at least, with some condition incident to terrestrial life, a conclusion further substantiated by the fact that the metamorphosis to the adult state in *Desmognathus* involves the development of a great multitude of glands of the granular type distributed over the entire surface of the body, in place of the few isolated groups of such glands with which the larva is supplied. Further, the fact that so closely related a form as *Spelerpes bilineatus* does not possess such glands while in the larval state leads one to ask what difference in environment or habits of these two species could serve to explain so decided a structural difference. We at once remember that the most essential difference lies in the fact that *Desmognathus fusca* is terrestrial in its egg laying habits while *Spelerpes bilineatus* is aquatic, and that consequently there is no terrestrial larval period in the latter species. Moreover, *Spelerpes* larvæ live in deeper pools than do the *Desmognathus* larvæ, and so far as I have observed, do not have the habit of lying upon leaves at the very surface of the water, which is so characteristic a position of the *Desmognathus* larvæ, and one which brings out of the water exactly those regions of the body which are supplied with the acinous glands, the mid-dorsal region of the trunk and the slightly elevated latero-dorsal surfaces of the head. Here again, then, there seems to be a decided connection between exposure to air and the function of the granular acinous glands. The solution of the problem is one which can be made only after extended experimental comparative study of different amphibians and must therefore be postponed until such study is completed.

The other set of specialized integumental organs, the neuro-

masts, are also prominent features of the skin during the entire larval life. They remain in practically the same condition except that as the skin becomes thicker and more compact, the accessory cells of the neuromasts become more numerous and more closely crowded together. Organs of this type are said to be associated with aquatic life and to be adapted to the reception of pressure stimuli. In view of this, it is interesting to note that the neuromasts are probably connected with the ready response of the larva both to a slight increase in the depth of the water above it, and to mechanical pressure applied to opposite sides of the body.

## Metamorphosis.

Metamorphosis occurs, as above stated, in late spring. The earliest date at which I have collected larvæ showing signs of the approaching change is May 11, the latest is June 17. There is strong evidence, however, in the histological conditions and body proportions of the small adults of from 33 mm. to 35 mm. in length which I have collected early in June, that they have only recently undergone metamorphosis, and adult specimens of similar size and proportions may be collected in early fall also (Table II. and Graphs III. and IV.). It thus seems probable that the period of metamorphosis may begin as early as May and may extend well into the summer, a range of time comparable to that known to exist for both the egg-laying and the hatching periods.

Larvæ which are undergoing metamorphosis are usually not found in the water but among the dead leaves and loose debris along the edge, often headed away from the water as if they were in the act of crawling out of it.

The noticeable changes in external appearance which take place during the process are the following:

1. The body proportions change, the head becoming relatively shorter and its posterior region narrower and the tail longer.

2. The median fin fold characteristic of the aquatic larval period undergoes atrophy, the cross section of the tail thus acquiring a more rounded contour.

3. The gill bushes gradually diminish in length, each filament becoming blunt at its extremity instead of slender and pointed,

and finally the gills entirely disappear, a process involving the final closure of the gill slits.

4. The whole gill region becomes shorter and narrower, the latter change being due in part to the flattening down of the prominent bulging supra-branchial regions so that this portion of the head is no longer the widest one.

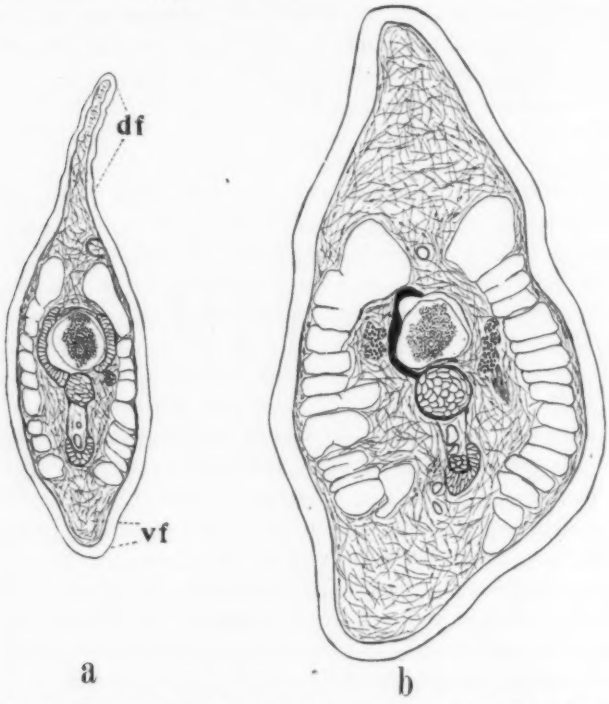



FIG. 16. Cross sections at the level of the 10th vertebra posterior to the cloaca showing (*a*) the typical aquatic larval form of tail with the dorsal (*df*), and the beginning of the ventral (*vf*) fin fold; (*b*) the change of form at the approach of metamorphosis. Note that (*b*) is cut slightly obliquely. Drawn with Abbé camera. × 60.

5. Further striking changes in the appearance of the head are due to the development of eyelids (*eyl*, Fig. 17) and of glandular masses in the orbit ventral to the eye, so that the eye and its surroundings come to possess the bulging appearance which is so characteristic an adult feature.

6. The naso-labial groove (*gr*, Fig. 17) makes its appearance, extending from the latero-ventral border of each external naris until finally, at the completion of metamorphosis, it has reached the border of the upper lip.

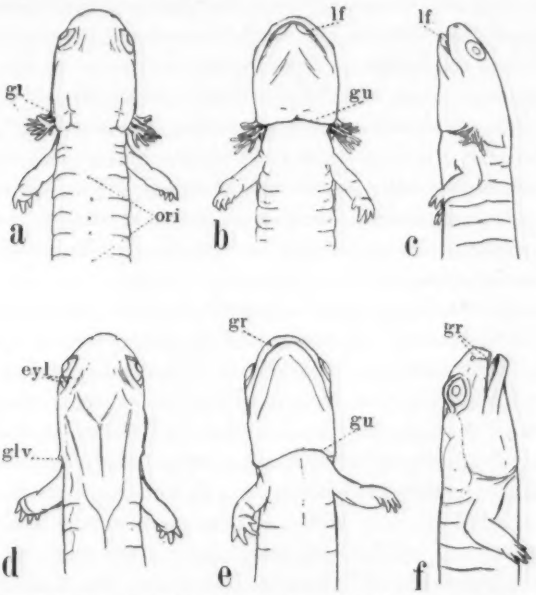



FIG. 17. (*a*), (*b*), and (*c*), dorsal, ventral, and lateral views respectively of the anterior region of the body of a *Desmognathus fusca* larva in which metamorphic changes have begun to appear; (*d*), (*e*), and (*f*), dorsal, ventral, and lateral views respectively of the anterior region of the body of a *Desmognathus fusca* in which the process of metamorphosis is just completed; *eyl*, eyelids, present only after metamorphosis; *gl*, gills which have become slightly blunted and shortened in (*a*), (*b*), and (*c*), and have disappeared altogether except for a slight vestige, *glv*, at the time of metamorphosis; *gr*, nasolabial groove, present only after metamorphosis; *gu*, gular fold; *lf*, labial fold which disappears at metamorphosis; *ori*, orifices of some of the larval acinous glands. Note that with metamorphosis the posterior region of the head becomes relatively narrower. Drawn with Abbé camera. × 4.25.

7. The labial folds (*lf*, Fig. 17) disappear, and the extent of the mouth opening increases, the lateral angles coming to lie as far back as the posterior angle of the orbit; thus a form of mouth opening adapted to the capture of larger prey, from a terrestrial as well as an aquatic medium, supplants the scooping or sucking apparatus of the aquatic larva.

8. The whole external surface gives evidence of the formation of a thin moult layer of epidermis, which here and there becomes separated from the deeper layers and thus presents a loose appearance.

In individuals in captivity these changes in external appearance have been observed to occur in the course of a few days during which time the individual crawls persistently out of the water. The metamorphosis may be considered quite complete when, in addition to the above enumerated changes in external appearance, the distinctive physiological metamorphic phenomenon occurs, and the animal, with mouth tightly closed, lowers the floor of the mouth and pharynx, and, drawing in air through the short nasal passages, fills its bucco-pharyngeal cavity and thus establishes aerial respiration.

Although the final steps in metamorphosis take place with great rapidity, the histological changes preliminary to the process begin considerably earlier. Thus specimens of larvæ collected in May show decided indications of the approaching change. The most striking of these indications is the large amount of mitosis which is in progress especially in the deeper layer of the epidermis (Plate V., 26, 27 and 29), resulting in so rapid a multiplication of the epidermal cells that the earlier arrangement in two fairly definite layers gives place to a considerable irregularity of arrangement of the cells of the deeper layer, the epidermis thus coming practically to consist of three layers. The arrangement of the cells of the outer layer remains unaltered, however, their cuticular borders forming, as before, a continuous external covering. The cells of the outer layer become, however, more and more flattened and are eventually to be cast off as the first moult layer, not, however, until after metamorphosis.

This flattening of the external layer of cells in part accounts for the actual decrease in thickness of the epidermis which becomes evident as the time of metamorphosis approaches; by far the greater cause of this, however, lies in the gradual disappearance of the Leydig cells, which by their turgor so greatly increase the thickness of the epidermis during early larval life. The invasion of these cells by pigment cells, connective tissue

cells and leukocytes during the larval period has already been noted. As metamorphosis approaches (Plate V., 28, 29, and 30), the nuclei of the Leydig cells (*lc*) become shrunken and the cell space is finally entirely given over to the intruding elements (*pgc*(*inv*) and *leu*) from the deeper region. The surrounding cells of the deeper epidermal layer, although increased in number by mitosis so as to form practically two layers instead of one, are correspondingly decreased in size and thus do not together equal in thickness the former diameter of the Leydig cells.

The thickness of the whole skin, *i. e.*, epidermis plus corium, does not become less; for coincident with the decrease in thickness of the epidermis there is a formation of loose vascular corium (*cor.l*) external to the dense corium and separating from it the deeper layer of epidermal cells which are in contact with it during the larval period. The formation of this loose corium is indeed inaugurated in the migration of leukocytes and connective tissue elements into the vacuoles of the Leydig cells and between the cells of the epidermis. But it is only with the breaking up of these Leydig cells in preparation for metamorphosis that these elements and other similar ones come to form a definite layer, and even then many of the connective tissue elements intrude themselves between the epidermal cells.

The development of integumental glands is particularly characteristic of the preparation for metamorphosis. These glands may be enumerated as follows: (1) General acinous glands; (2) naso-labial glands; (3) orbital glands.

The acinous gland anlagen appear during the general premetamorphic mitotic period of the epidermis (Plate V., 29 and 30). They arise from the deeper layer of the epidermis and may first be distinguished as little groups of from four to eight cells with large nuclei, some of which are usually in the process of mitosis (*ac*, Plate V., 29). They are abundantly distributed over practically all regions of the body, although somewhat less numerous in the skin covering the appendages. They rapidly increase in size and assume the form of hollow spherical acini, each with a definite lumen which opens to the exterior by a narrow duct (Plate V., 30). The bodies of the glands intrude into the loose corium and thus lie below the level of the epidermis,

which is, however, thin in the region immediately surrounding the gland. Embedded thus in the loose vascular corium, each gland comes to be surrounded by a capillary ring (*cap*), which, joining similar rings about the neighboring glands, forms a part of the rich capillary network of the skin. These glands and their ducts, the latter slender and lined with thin cells which are continuous with the cells of the external layer, are fully formed at the time of metamorphosis and before the first moult (*ml*, Plate VI., 31) occurs. The nature and function of their secretion and the methods by which they are replaced must be left for further and more extended investigation. Suffice it to say that many, if not all of them, show from the first a striking similarity in structure to the relatively larger and less numerous acinous larval glands above described. One anatomical distinction between the larval and adult glands must, however, be clearly understood, namely, that the larval glands lie wholly *below* the level of the dense corium, the ducts alone passing through this to reach the external surface. The larval glands remain functional up to the time of metamorphosis, and thus in the metamorphosing larva both the larval and the adult sets of glands are present, the former beneath the dense corium, the latter external to it within the loose corium (cf. Fig. 15, *e*). After metamorphosis, however, the larval glands collapse and lose their connection with the external surface, their cells undergo atrophy, and the glands rapidly disappear. It is this atrophy of the supra-branchial group of glands which results in the marked narrowing of this region of the head at metamorphosis (cf. Fig. 17).

The naso-labial glands consist of a series of tubular glands opening in close proximity to the external nasal orifices and along the border of the naso-labial groove (Whipple, '06). Of these glands (many of which become very extensive in the fully developed adults) the two largest ($nl_1$ and $nl_2$, Figs. 18, 19, and 20) begin their development before metamorphosis, and at the time of metamorphosis have attained a size and condition indicative of their functional importance in adult life. The one of these which opens at the dorsal angle of the nasal orifice extends, at this time, beneath the skin as far posteriorly as the anterior angle of the orbit, is somewhat convoluted, and branches near

its distal (posterior) end; the other gland, which opens near the ventral angle of the naris, is also well developed, extends nearly as far posteriorly as the first but in a more ventral location, and as yet shows no branching. Although other glands of the

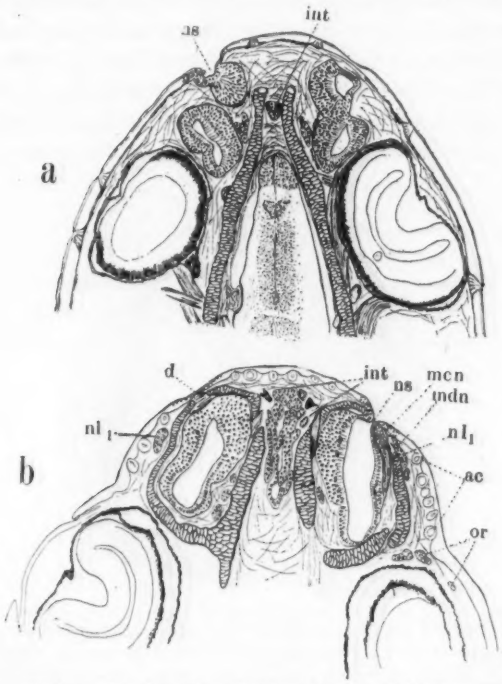



FIG. 18. Horizontal sections through the nasal region of (*a*) *Desmognathus fusca* larva collected in February, and (*b*) an individual immediately after the completion of its metamorphosis, for comparison of the glandular development; *ac*, acinous glands of adult stage; *d*, duct of the most dorsal of the naso-labial glands ($nl_1$); *int*, intermaxillary glands; *mcn*, and *mdn*, musculus constrictor naris, and musculus dilatator naris respectively; $nl_1$, the first nasolabial gland; *ns*, external naris; *or*, orbital glands. Drawn with Abbé camera. × 32.5.

group are slightly developed, none of the tubules have as yet become so extensive as to enter either the premaxillary foramina or the grooves of the maxillary bones as they are to do later in the fully developed adult. Simultaneously with the development of these naso-labial glands the muscular apparatus for opening

and closing the naris develops. This consists of a constrictor and a dilatator muscle (*mcn* and *mdn*) and forms an effective apparatus very characteristic of adult amphibians (Bruner, '96 and '01, I. W. Wilder, '09).

The naso-labial glands have to do with the terrestrial life into which the process of metamorphosis introduces the animal, in that they not only keep pliable the thin crescentic fold which by means of the contraction of the constrictor muscle closes the external naris against the entrance of foreign bodies, such as dirt when the animal is burrowing, but they also appear to have the peculiar

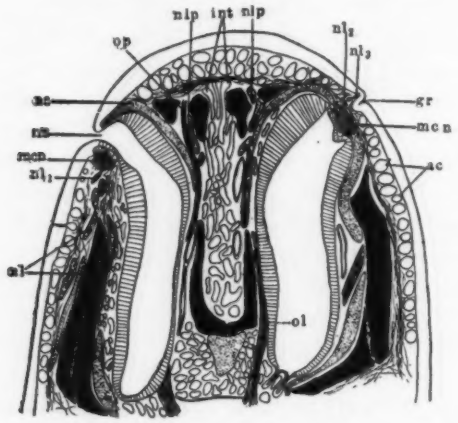



FIG. 19. Horizontal section through head of adult *Desmognathus fusca*, × 20. *Ac*, acinous glands of skin; *gr*, cross-section of naso-labial groove; *int*, intermaxillary glands; *mcn*, M. constrictor naris; *nc*, nasal capsule; *nl*, naso-labial glands; $nl_1$, $nl_2$, $nl_3$, the first, second and third naso-labial glands respectively; *nlp*, tubules of naso-labial glands within the premaxillary foramina; *ns*, external naris; *ol*, olfactory nerve; *op*, internal nasal branch of ophthalmic nerve.

function of so repelling water from the surface of the skin immediately surrounding the naris and bordering the naso-labial groove that the latter may, by capillary action, almost instantaneously drain off the tiny drop which fills the nasal depression after a temporary immersion in water. Thus this water is prevented from entering the nasal passage when the animal, upon emerging from the water, reopens the nares by withdrawal of the crescentic fold. The nasal passages are by this means kept dry and fully

ready for the immediate resumption of aerial respiration (Whipple, '06). It is a significant fact, therefore, that at the completion of metamorphosis this naso-labial apparatus is in working order.

The orbital glands lie ventral to the eyeball (*or*, Fig. 21, *b* and *d*)and open along the depression between the inner surface of the lower eyelid and the eyeball itself. They are tubular glands and make their appearance simultaneously with the formation of the fold which gives rise to the lower eyelid itself, shortly before metamorphosis.

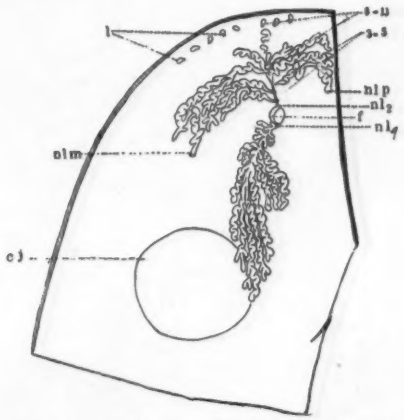



FIG. 20. Dissection of adult *Desmognathus fusca* in which the skin is removed from the right side of the head, the naso-labial glands being removed with it. Drawing shows under surface of skin with glands in place. *Cj*, conjunctiva; *l*, labial glands; 3–5 and 8–11 indicate the enumeration of the naso-labial glands; *nlm*, cut end of tubule which lies in the groove of the maxillary bone. Other designations as in Fig. 19.

In connection with the development of the orbital glands it should here be noted that unlike all other Urodeles (so far as the facts have been reported), *Desmognathus* has no naso-lacrimal ducts. As these are present in so closely related and associated forms as *Spelerpes* and *Plethodon*, as well as in the less closely related lunged forms, their absence here has some significance which demands further study of the comparative morphology and the habits of this species.

In addition to the integumental glands, the development of two other sets of glands must be noted among the premetamorphic changes, the intermaxillary and the lingual glands. The former is an unpaired gland and is present indeed throughout larval life (*int*, Fig. 18) as a single, slightly convoluted tube

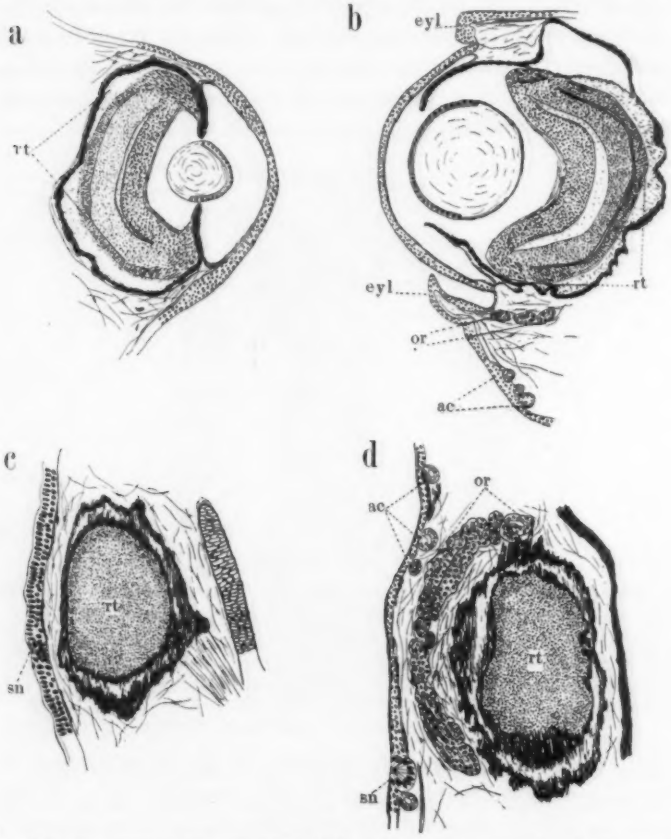



FIG. 21. Sections for comparison of the eye and its surroundings in the larval and adult stages of *Desmognathus fusca*; (*a*) transverse section through the left eye of a larva; (*b*) transverse section through the right eye of a recently metamorphosed adult; (*c*) horizontal section through the ventral region of the eye of the larva; (*d*) horizontal section through the ventral region of the eye of a recently metamorphosed adult. *Ac*, acinous glands; *eyl*, eyelids; *or*, orbital glands; *rt*, retina; *sn*, sense organs of the integument. Drawn with Abbé camera. × 60.

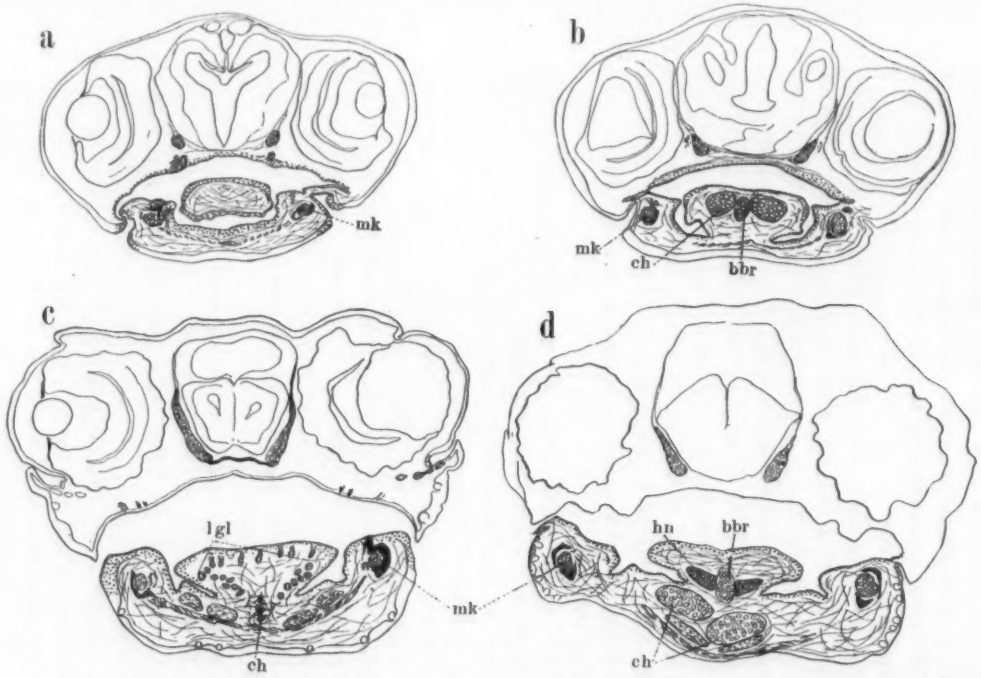



FIG. 22. Cross sections through the heads of (*a*) *Desmognathus fusca* larva at the level of the free anterior region of the tongue, (*b*) the same larva at the level of the articulation of the ceratohyals and basibranchial (cf. Fig. 25, *a*), (*c*) recently metamorphosed adult at a level corresponding to (*a*), and (*d*) the same adult at the level corresponding to (*b*) (cf. Fig. 25, *b*); *bbr*, basibranchial; *ch*, ceratohyal; *hn*, accessory horns; *lgl*, lingual glands; *mk*, Meckel's cartilage. Drawn with Abbé camera. × 32.5.

leading dorsally from the roof of the mouth, from a point a little anterior to the posterior nares. During the premetamorphic period of mitosis and glandular development, the intermaxillary gland becomes enormously enlarged and complicated and fills the space between the two maxillary bones with its mass of convoluted tubules.

The tongue possesses no multicellular glands until, with the approach of metamorphosis, large numbers of tubular glands (*lgl*, Fig. 22, *c*) appear opening in the more distal region of the tongue upon its dorsal surface, and transforming the tongue into a glandular mass. The development of both intermaxillary and lingual glands at this time is, of course, suggestive of a preparation for the terrestrial life of the adult. By means of their secretions the mouth is kept moist both for its respiratory function and to render more sure the capture and retention of terrestrial prey.

Among the most significant of the structural changes involved in metamorphosis are those concerned in the atrophy of the gills and the modifications of their associated structure, the visceral skeleton. Externally changes do not occur in the gill bushes themselves until within a few days of the completion of metamorphosis. Then the filaments become shorter and more rounded, and the whole structure undergoes rapid shrinkage and atrophy. As the gills are merely blood vessels covered with a thin layer of epidermic cells, the withdrawal of the blood from them so reduces them in size that the process of atrophy can take place very quickly.

The metamorphic changes in the visceral skeleton consist mainly in the atrophy of the distal end of the first epibranchial and the atrophy of the whole of the second, third, and fourth epibranchials (Figs. 23, 24, and 25). The process begins at the time of the premetamorphic mitotic period, and appears first in the distal ends of the epibranchial cartilages where the hyaline matrix becomes dissolved around the groups of cartilage cells (Fig. 23, *b*). With the atrophy of the free ends of the epibranchials, there occurs that shortening of the gill region which is mainly responsible for the decrease in the proportionate length of the head as the animal passes from larval to adult life. Simul-

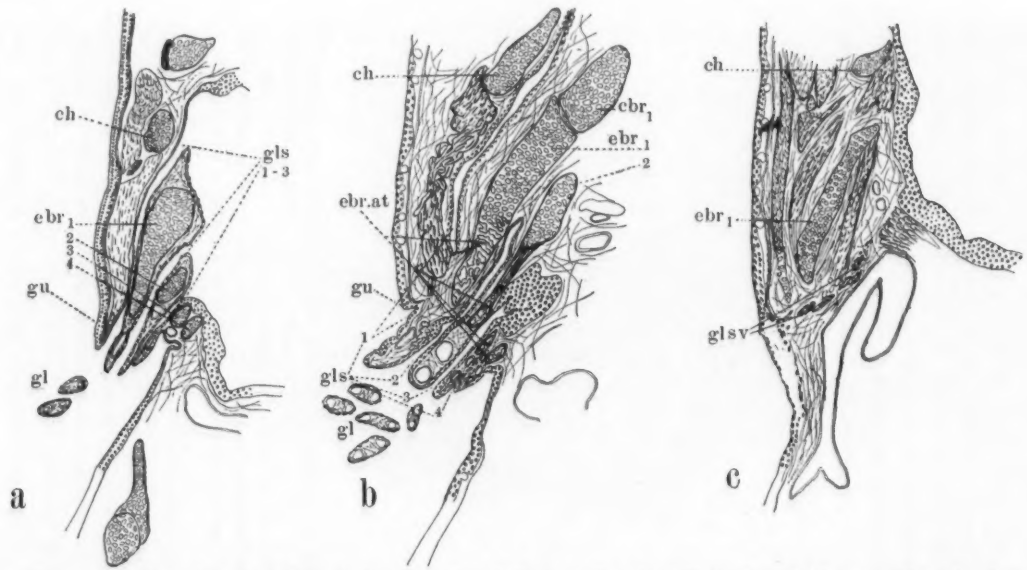



FIG. 23. Horizontal sections through the gill region of *Desmognathus fusca* illustrating the changes which take place at metamorphosis; (*a*) aquatic larva collected in February, showing the typical larval condition of the region; (*b*) larva in which metamorphic changes have begun (cf. Fig. 17, *a*, *b*, and *c*); (*c*) an individual which has just completed its metamorphosis (cf. Fig. 17, *d*, *e*, and *f*); *ch*, ceratohyal cartilage; *cbr*1, first ceratobranchial cartilage; *ebr 1*, *2*, *3*, and *4*, epibranchial cartilages; *ebr.at*, regions of the epibranchials which are undergoing atrophy; *gl*, gills; *gls*, gill slits; *glsv*, vestiges of the gill slits; *gu*, gular fold. Drawn with Abbé camera. × 32.5.

taneously with the atrophy of the epibranchials there occurs an atrophy of the whole of the second basibranchial except its extreme posterior end, which persists and later ossifies to become a little bifurcated structure designated by Wiedersheim ('77) as the thyreoid bone (*bbr 2*, Fig. 25, *b*). With the atrophy of the second basibranchial cartilage, which during larval life forms one continuous chondrification with the first pair of ceratobranchials, the latter become separated from each other and each articulated

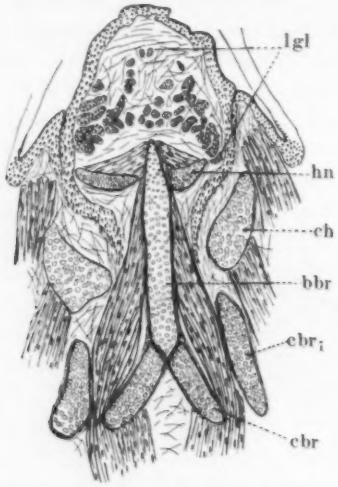



FIG. 24. Horizontal section through the tongue and hyo-branchial apparatus of a recently metamorphosed adult *Desmognathus fusca* (cf. with the drawing of the whole apparatus shown in Fig. 25, *b*); *bbr*, basibranchial; *cbr 1* and *2*, first and second ceratobranchials; *ch*, ceratohyal; *hn*, accessory horns for the support of the adult tongue; *lgl*, lingual glands. Drawn with Abbé camera. × 32.5.

with the first basibranchial. In the two or three final days before metamorphosis the medial ends of the ceratohyals become detached from the anterior ends of the first basibranchial (*ch*, Fig. 22, *b* and *d*), while posterior to their former point of attachment, a pair of cartilaginous processes grow out from the basibranchial to serve apparently as an additional support for the tongue (*hn*, Figs. 22, *d*, 24, and 25).

The final change which completes the atrophy of the gill region is the closure of the gill slits of which there are four upon

each side, the most anterior being the extensive one anterior to the first gill arch and beneath the gular fold. Previous to metamorphosis the gular fold becomes noticeably shortened, keeping pace with the shortening of the cartilaginous arches.

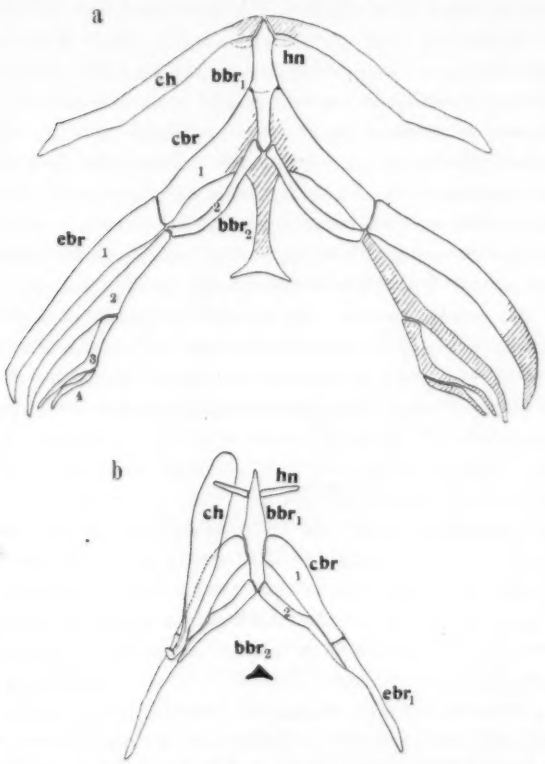



FIG. 25. (*a*) A dorsal view of the hyo-branchial apparatus of an aquatic larva collected in mid-winter, showing the typical larval structure. The shading indicates the regions which will atrophy at metamorphosis. The dotted lines indicate the regions where the accessory horns (*hn*) will develop at the time of metamorphosis. *Bbr*1 and *bbr*2, first and second basibranchials; *cbr*1 and *cbr*2, the first and second ceratobranchials; *ch*, ceratohyal; *ebr 1*, *2*, *3*, and *4*, the four epibranchials. Drawn with Abbé camera from a preparation stained *in situ* with methylene blue, and cleared with clove oil. × 40.

(*b*) A dorsal view of the hyo-branchial apparatus of an adult *Desmognathus fusca*. Designations as in (*a*). Note the persistence and ossification of the posterior end of the second basibranchial, forming the *os thercoideum* of Wiedersheim ('77). Drawn with Abbé camera from a dissection. × 6.

With the final withdrawal of the blood from the gill bushes, the latter lie against the lateral wall of the pharyngeal region like a temporary operculum, and rapidly fuse with the epidermis covering the surface over which they lie, thus effecting the external closure of the gill slits (Fig. 23, *b* and *c*).

For some time after metamorphosis the whole region is in a disorganized condition histologically, and sections show beneath an external covering of the thinnest of epidermic layers, a great meshwork of distended blood vessels, together with the remnants of epithelial lining of gill slits, which through the agency of the ever active leukocytes is rapidly disintegrating.

The transition from larval to adult life involves in *Desmognathus* no fundamental change in the nature of the food, since throughout the whole life of the species the food consists mainly of living animal forms. As would be expected, therefore, metamorphosis necessitates no noticeable changes in the digestive apparatus, as it does in the case of those amphibians in which the nature of the food changes at metamorphosis from vegetable to animal.

## Adult Stage.

With regard to the growth and development of *Desmognathus* subsequent to metamorphosis I can give no very exact data. The many adults which I have measured range from 29 mm. to 104 mm. in length, the former showing, of course, unmistakable evidences of recent metamorphosis. Specimens collected at any one time do not show sufficient evidence of falling into distinct groups as to size to suggest definite yearly amounts of growth, with the possible exception of the smaller sizes which, in the case of those collected in the late spring, for example, fall into groups averaging 33 mm., 43 mm., and 56 mm. in length, the former recently metamorphosed, and the others presumably adults of one and two years' growth, respectively. Since the proportionate lengths of body regions of the smallest adults show a much wider range of variation as well as a closer similarity to the proportions of the larvæ than to those of adults of 55 mm. and over, it seems evident that not only is the growth during the first year or two more rapid but that the change of length pro-

portions from those at metamorphosis to those of the final adult life, a change which involves a relatively more rapid growth of the caudal than of the other regions, also takes place more rapidly during the first two years (cf. Table II., Graphs III. and IV.). All of the adults, however, show a slight range of variation in the proportions of length of body regions which is in general correlated with the size of the specimens, as if the caudal region continued always to grow slightly more rapidly than the rest of the body. Here, however, the actual facts are sometimes masked by the frequency of regeneration of the tail, since a large percentage of adults show the tail to be in some stage of regeneration. I have seen no evidence that this loss of tail is due to self-mutilation as in the case of *Plethodon*, although the natural supposition would be that such is the case. Such regenerating specimens have, when detected, been excluded from my statistics, but when the process of tail regeneration is nearly complete, it is not always possible to decide whether the specimen presents a case of regeneration or one at the lower limit of normal variation. The question of the time of sexual maturity may also throw some light on the duration of the period of the more rapid growth of adults. At the time of metamorphosis the two sexes may be distinguished by the histological condition of the germ cells, some of the ova having already entered upon the growth period. Small adults, up to the 43 mm. stage, *i. e.*, presumably of a year's growth as adults, give no indication of sexual maturity. I have had too little opportunity to examine in this particular, specimens of the next larger size, presumably of two years' growth, to be able to state definitely that they may not be sexually mature at this time, but the few that I have examined were not so. On the other hand, specimens of 68 mm. and over of both sexes are sexually mature, apparently after three years of adult life. In the mating season the lips of the cloaca are often somewhat everted, and the two sexes may be distinguished by the fact that the female cloacal lips display numerous folds which converge anteriorly, with an anteriorly projecting papilla in the mid-line at the posterior angle of the two lips. In the end of this papilla, which lies within the cloacal cavity, is the orifice of the spermatheca. The lips of the male cloaca are thickly covered with villi-like processes.

The changes in structure during adult life are otherwise those of the natural growth of the various organs. Thus, although the epidermis retains the condition reached soon after metamorphosis of some two or three layers of cells beneath the moult layer, the acinous glands increase constantly in size and number, and the loose corium in which they are embedded, as well as the dense corium beneath, becomes correspondingly thicker and the whole skin more deeply pigmented and more vascular. So also the other integumental glands, such as the naso-labial and the orbital, become more numerous and their tubules more convoluted and longer, while the naso-labial groove itself grows deeper and more clearly marked.

The muscular development keeps pace with the general increase in size. Thus the power and rapidity of motion which may be observed in all stages of this species becomes very pronounced in the large adults. The long muscular tail plays an important rôle in locomotion, acting as a strong propelling organ as it strikes against the ground first upon one side, then upon the other, thus sending the body forward. This method of locomotion is of course a part of the adaptation to a burrowing habit.

The following list of the contents of the stomachs of 18 specimens of adult *Desmognathus* of different sizes and collected at different times and from different localities will serve to illustrate the variety of food materials which are made use of. It will be noted that the food is wholly animal and that the list includes aquatic as well as terrestrial forms.

Specimen No. 1. Moulted skin of *Desmognathus* (presumably his own).

No. 2. A caddice fly, and three dipterous larvæ.

No. 3. A small adult dipterous insect.

No. 4. Unidentifiable animal fragments.

No. 5. Insect larva, unidentified.

No. 6. Spider, probably an *Azalena*, with egg mass.

No. 7. Large black ant.

No. 8. Fragments of beetles, hymenopterous insects, and sowbugs.

No. 9. Two small beetles, a dipterous insect, a small green caterpillar, and an insect larva.

No. 10. Remains of an aquatic insect larva.

No. 11. Sand, dirt, and unrecognizable debris.

No. 12. A small annelid, fragments of a larger annelid, two small crustaceans (probably *Gammarus*), some mites, and fragments of adult insects.

No. 13. An adult dipterous insect, fragments of other small insects, some very minute mites, and unrecognizable debris.

No. 14. A small mite.

No. 15. A whole *Desmognathus* larva.

No. 16. A whole *Desmognathus* larva.

No. 17. Moulted skin of *Desmognathus*.

No. 18. An earthworm, a small spider, and some six or eight specimens of *Gammarus*.

The breathing habits of adult *Desmognathus* deserve especial discussion because of the lungless condition of the animal. As in all amphibians the skin is a most efficient breathing organ, and so long as it is kept in the normal moist condition which the burrowing habit of the species insures, it probably furnishes ample means for the aeration of the blood which circulates through its capillary network. As is usual with amphibians also, the bucco-pharyngeal cavity is made use of to supplement the cutaneous respiration. Bucco-pharyngeal respiration is accomplished by a rapid fluctuation of the floor of the tightly closed mouth, which results in a rapid succession of movements of air in and out through the wide-opened nares. Correlated probably with the lungless condition, is the fact that these fluctuations are far more rapid in *Desmognathus* than in the lunged forms; moreover, *Desmognathus* in common with other lungless forms (*Spelerpes* and *Plethodon* for example) possesses a relatively longer œsophagus provided with muscles by means of which it may be held distended, thus increasing the size of the respiratory surface by the addition of a mucous membrane which was shown by the researches of H. H. Wilder ('01) and Seelye ('06)

to be supplied with a rich capillary network, rendering it an efficient breathing organ.

As I have shown elsewhere (Whipple, '06), the lungless salamanders do not have the habit, otherwise common to amphibians, of changing from aerial to aquatic bucco-pharyngeal respiration when in the water, even though they may be kept completely immersed for several days. Under these conditions the external nares are kept tightly closed and the floor of the mouth forcibly drawn in. The immersed condition is of course an unnatural one for *Desmognathus* as is shown by its frantic efforts to escape from the water, but it shows nevertheless no physical ill effects of a prolonged imprisonment beneath the surface, a proof that the skin is under these conditions a perfectly adequate breathing organ.

In his paper on *Desmognathus fusca* and *Spelerpes bilineatus*, H. H. Wilder ('99) pointed out the great possibilities which are presented by both *Desmognathus* and *Spelerpes* as objects for laboratory study. The abundance and widespread occurrence of the two genera, the ease with which they may be collected in both the larval and the adult states throughout the entire college year, and the convenience with which they may be kept alive in the laboratory for months, with practically no care, render the material always available and inexpensive. The following account taken from Wilder ('99) gives valuable suggestions as to methods of keeping such material in the laboratory:

"*Method of Rearing in Confinement.*—The adults of both species, because of their peculiarities in respiration and the consequent necessity of keeping their skin moist, cannot be kept either in water or in a dry atmosphere, but may easily be kept for months or years in an ordinary fernery where the atmosphere is constantly saturated with moisture. I have in my laboratory a large fernery or terrarium, about 2 × 3 feet square and 2 feet high. The bottom consists of a zinc tray, 8 inches deep and water-tight. The top and sides are of glass and the front side runs in a frame with weights, being thus capable of being raised and lowered like an ordinary window-sash. In the bottom of this there are about 6 inches of good garden soil, in which are planted ferns and other wood plants. The surface is partly

covered with moss, and here and there are placed several stones, the size of one's fist, and a few pieces of rotten stump, arranged so as to give shelter to the adults. In one corner a crystallizing dish is sunk to the level of the soil. This is filled with water and the bottom covered with a little fine sand. Some duckweed, or *Salvinia*, may be placed upon the surface, and a few small stones should be put in a dish. At the beginning of the season, after arranging everything as above, enough water is poured in to drench the soil, and the sunken dish is filled. After this the terrarium is self-regulating. The water that evaporates is re-precipitated as moisture, and the total loss from the little pond in the corner is so slight that it needs replenishing not oftener than once in six months. If the terrarium is to support many animals, it is better to place a few earthworms, myriapods, etc., in it; and if the pond is designed for the rearing of larvæ, supplies of *Entomostraca* and a little *Spirogyra* to feed them with should be occasionally introduced. I have tried placing tiny bits of meat in prominent places, but they merely mould and have to be removed. I have kept as many as 20–30 adults and a dozen larvæ in my terrarium during an entire college year, and several times, on clearing it out in the fall after the summer vacation, I have found alive and in good condition adults which I had been unable to find in the spring, when I intend always to remove the animals. It seems most probable that these salamanders find enough to eat among the worms and insects introduced with the earth and plants, as they always appear in perfectly normal condition and contrast very forcibly with *Diemyctylus*, which grows thin and often starves to death when placed under the same conditions."

I might add to this account the fact that I have found very convenient for the accommodation of a small number of specimens, such terraria as may be readily constructed from cylindrical anatomical jars of heavy glass, measuring from 8 to 18 inches in diameter and from 6 to 10 inches high, and provided with a tightly fitting but easily removable glass cover. Such jars, equipped with earth, stones, and plants as above directed by Wilder, will accommodate according to size from two to ten specimens of adult *Desmognathus* and are small enough to be

easily moved about. Moreover, owing to the fact that the adult *Desmognathus* occasionally feeds upon the larvæ, I have found it safer for the latter to be kept in separate shallow receptacles, such as glass crystallizing dishes, equipped with a little earth covered with water to the depth of a few centimeters, and containing a quantity of decaying, slime-covered leaves which have been transported to it from the natural habitat of the larvæ. Such an arrangement, if kept tightly covered to prevent evaporation, will support the larvæ in a normal condition for months without further care.

The usefulness of *Desmognathus* as material for the various phases of introductory research work cannot be over-emphasized. As Wilder has pointed out, the large size and the unpigmented nature of the egg render it excellent material for the study of general amphibian embryology. The small size of the larvæ (from 15 to 30 mm. in length), and their long continuance in the larval state, make them particularly valuable material for the introduction to methods of study by serial sections, since the specimens are not only small enought to decalcify easily and section beautifully but are large enough and enough like the adult in their general anatomy to also lend themselves readily to study by dissection, and by the numerous methods of injecting and selective staining followed by clearing *in toto*. Studied in this way it furnishes, pedagogically, an excellent means of transition from the study of the gross anatomy of larger vertebrate forms to the study both of their histology and their earlier embryology. Thus it bridges a gap which it has often been customary in college courses to make at a jump which too often lands even the able student in a region of mystery. As material for the study of adult anatomy, also, *Desmognathus* is a convenient form, the larger specimens being quite large enough for ready dissection, and not too large to use with the dissecting microscope for finer details, while both the large and the small adults may be readily decalcified and sectioned for the microscopic study of their structure. Moreover the species with its succession of terrestrial and aquatic stages, its lungless condition and other peculiarities of structure, presents a number of little physiological and anatomical problems, the working out of which

is not too difficult for the inexperienced beginner, and may even yield important contributions to our general knowledge.

It is with the hope, therefore, that these pages will be useful and suggestive to those interested in any amphibian problems upon which the study of *Desmognathus* might have some bearing, that it has seemed worth while to collate the known facts concerning the life history of this common and interesting species.

SMITH COLLEGE, NORTHAMPTON, MASS.,
June 26, 1912.

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LIST OF ABBREVIATIONS USED IN THE PLATES.

*ac*, acinous glands.
*cap*, capillaries.
*cl*, columnar epidermal cells.
*cor.d*, dense corium.
*cor.l*, loose corium.
*cu*, cuticle formed by the outer borders of the outer layer of epidermal cells.
*d*, duct of acinous gland.
*ep*, epidermis.
*ext*, outer layer of epidermal cells.

*gc*, giant cells of the acinous glands.
*int*, inner layer of epidermal cells.
*intr*, intrusive cells (Schaltzelle), which project from the deeper layer into the outer layer.
*lc*, Leydig cells.
*leu*, leukocytes.
*lu*, lumen of gland.
*ml*, moult layer.
*mt*, mitosis.
*mt(inv)*, mitosis in a cell which has invaded a Leydig cell.
*pg*, pigment.
*pgbr*, branches of pigment cells.
*pgc*, pigment cells.
*pgc(inv)*, pigment cells which have invaded the Leydig cells.
*pgg*, intracellular pigment granules.
*sn*, integumental sense organ.
*sp*, spireme.
*yg*, yolk granules.

## Description of Plates.

Plates I., II., and III. A series of outlines drawn with Abbé camera, magnification × 4.25, showing various stages of *Desmognathus fusca* from the time of hatching to the attainment of practically adult proportions.

Plates IV., V., and VI. Sections of the skin of *Desmognathus fusca*, showing the structures characteristic of the various periods and the developmental changes which take place in the transition from one stage to another. Drawn with Abbé camera. × 450.

## Errata.

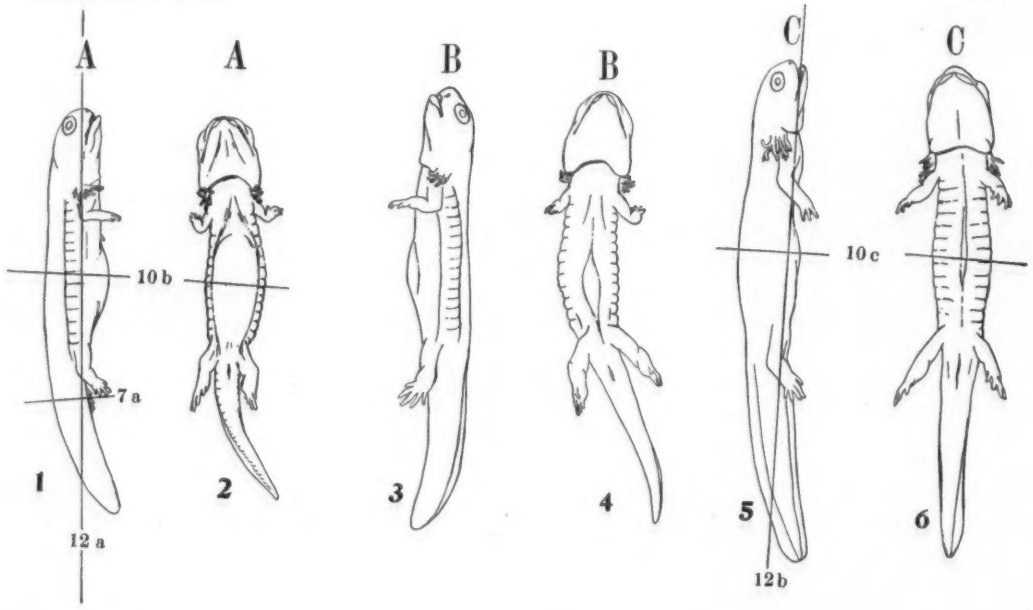
References throughout the text to Pl. I., Fig. 7–11, apply to Pl. II.

PLATE I.

FIGS. 1 and 2. Lateral and ventral views of terrestrial larval stage *A*, immediately after hatching. Lines *7a*, *10b*, and *12a* show the locations of the sections shown in the text figures correspondingly numbered.

FIGS. 3 and 4. Lateral and ventral views of terrestrial larval stage *B*, age 3 days.

FIGS. 5 and 6. Lateral and ventral views of terrestrial larval stage *C*, age 3 days. Lines *10c* and *12b* show the locations of the sections shown in the text figures correspondingly numbered.



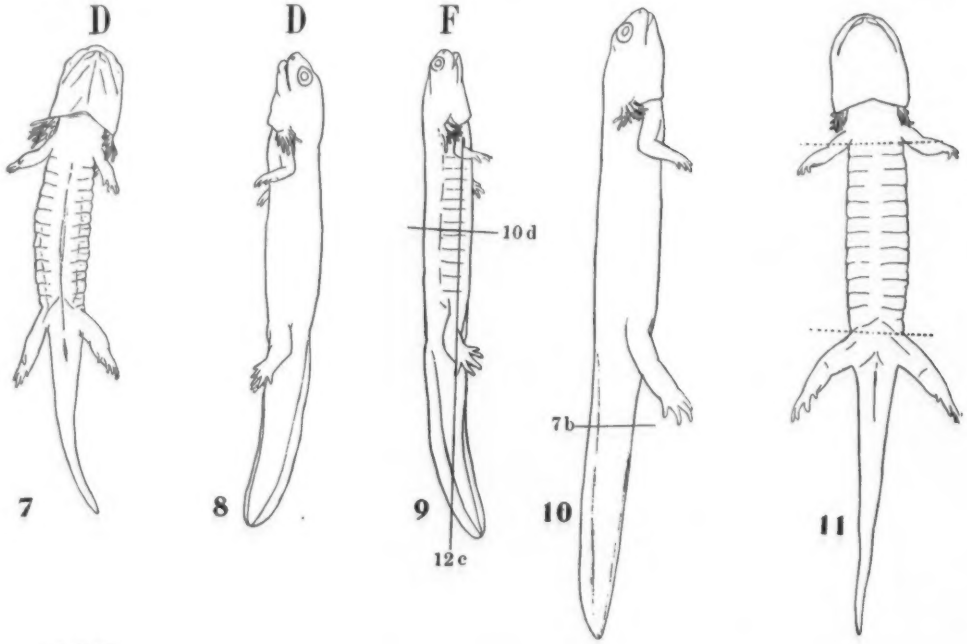

I. W. WILDER.

PLATE II.

FIGS. 7 and 8. Lateral and ventral views of terrestrial larval stage *D*, age 7½ days.

FIG. 9. Lateral view of terrestrial larval stage *F*, a small specimen which has attained the proportions of the aquatic larvæ, age 15½ days. Line *10d* and *12c* show the location of the sections shown in the text figures correspondingly numbered.

FIGS. 10 and 11. Lateral and ventral views of an aquatic larva collected in October. Line *7b* indicates the location of the section shown in the text figure correspondingly numbered. The dotted lines show the levels used in arbitrarily dividing the body into head, trunk, and tail regions for measurements (cf. Tables I., and II., and Graphs I., II., III., and IV.).




L. W. WILDER.

PLATE III.

FIGS. 12 and 13. Lateral and dorsal views of an aquatic larva collected in May, and hence nearly ready to undergo metamorphosis.

FIGS. 14 and 15. Lateral and dorsal views of a small, recently metamorphosed adult, collected in June.

FIG. 16. Ventral view of an adult 49.4 mm. long.

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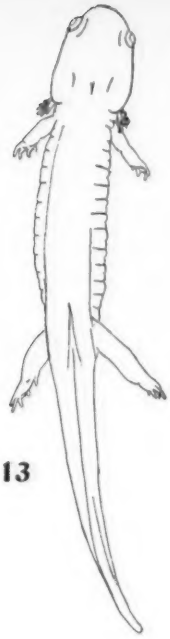

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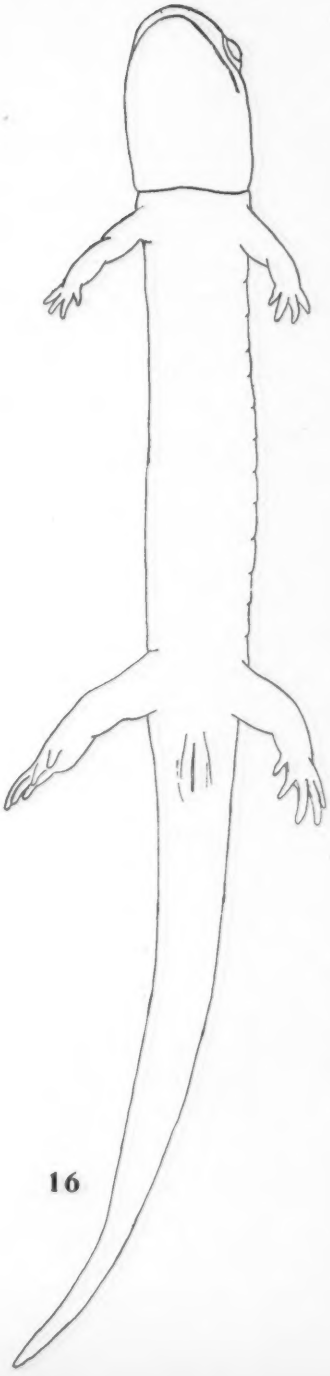

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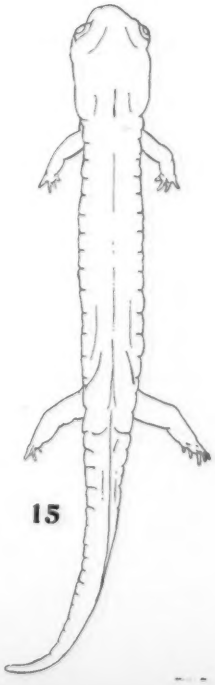

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## PLATE IV.

FIG. 17. Vertical section through the skin of the dorsal surface of a 13 mm. embryo of 30 days' development, showing the characteristic two layers of epidermal cells.

FIG. 18. Vertical section through the skin of the lateral surface of a 13 mm. embryo.

FIG. 19. Vertical section through the skin of the dorsal surface of a newly hatched terrestrial larva, stage *A*. Note the presence of a corium of dense connective tissue beneath the two layers of epidermal cells, the absence of mitosis in the epidermis, and the development of an external cuticle from the outer borders of the outer layer of cells.

FIG. 20. Vertical section through one of the integumental sense organs in the region of the gular fold, terrestrial larva, stage *A*.

FIG. 21. Section parallel with the external surface, through the outer layer of epidermal cells, dorso-lateral surface, terrestrial larva, stage *A*.

FIG. 22. Similar section through the inner layer of epidermal cells, dorso-lateral surface, terrestrial larva, stage *A*.

FIG. 23. Vertical section through the skin of the dorso-lateral surface of an aquatic larva collected in September. Note the presence of numerous Leydig cells in the deeper layer.

FIG. 24. Section parallel with the external surface, through the outer layer of epidermal cells from a lateral, unpigmented region of the body of an aquatic larva collected in September.

FIG. 25. Similar section through the inner layer of epidermal cells, lateral region, of an aquatic larva collected in September.

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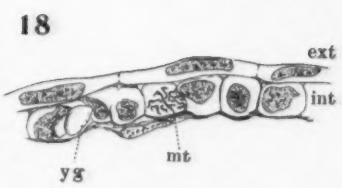
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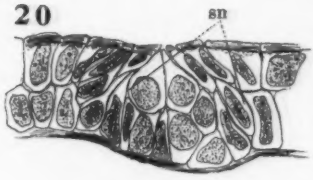
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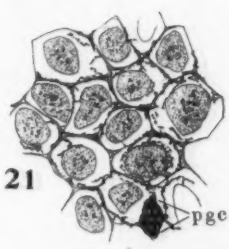
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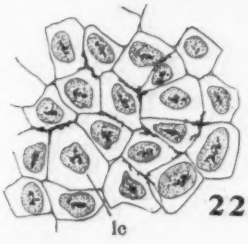
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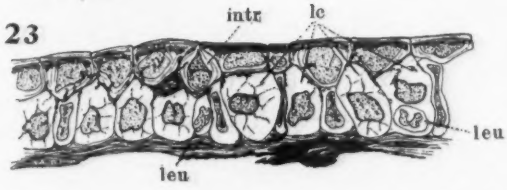
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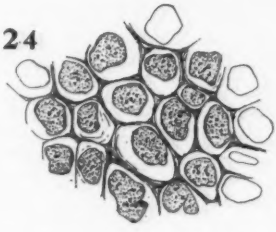
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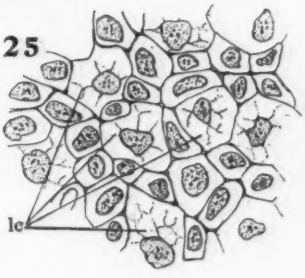
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PLATE VI.

FIG. 31. Vertical section through the skin of an individual which has just completed its metamorphosis, but in which the first moult has not yet occurred. Note the presence of a deeper layer of flattened cells which will become the next moult layer.

FIG. 32. Vertical section through the skin of a very small adult (length 29 mm.) collected in June shortly after metamorphosis and after the first moult has been cast.

FIG. 33. Vertical section through the skin of a large adult, showing the large size of the acinous glands, the great thickness of the loose corium, and the relative thinness of the epidermic layer (cf. larval condition).

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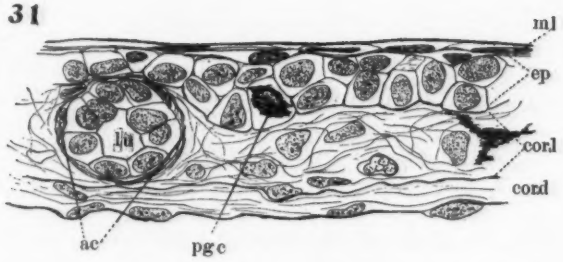
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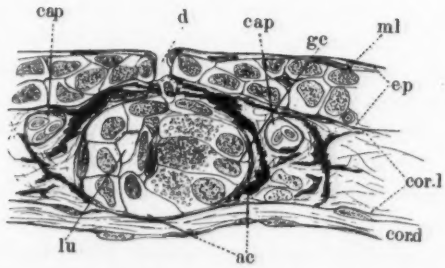
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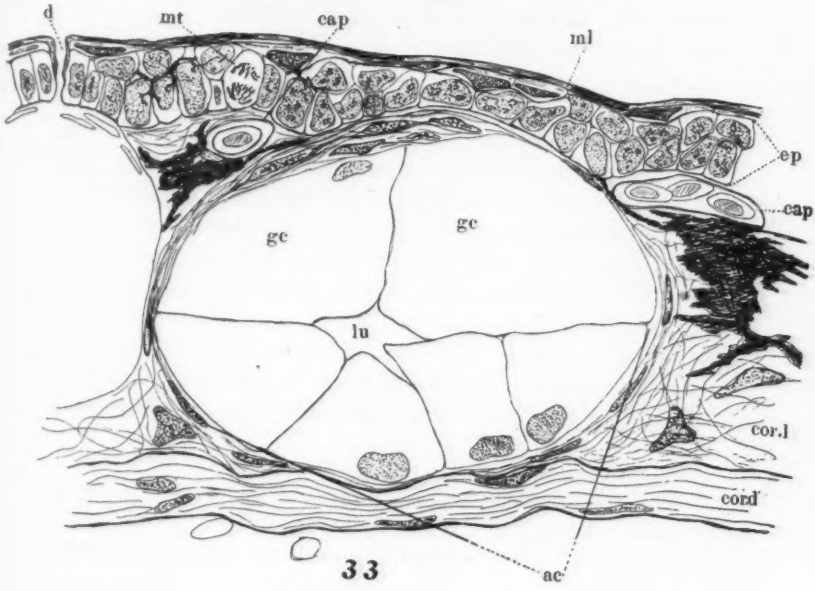
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# STUDIES IN ARTIFICIAL PARTHENOGENESIS.[1]

## I. Membrane Elevation in the Sea-Urchin Egg.

LEWIS V. HEILBRUNN.

*Introduction.*—The ultimate goal in the study of artificial parthenogenesis is the discovery of the chemical and physical forces which are assumed to cause the initiation of development. The pioneer workers on the problem (Tichomiroff, O. and R. Hertwig, Morgan) were content to regard the phenomenon as the result of a stimulus which arouses the egg from its resting condition. But the present tendency, thanks to the work of Loeb and his school, recognizes that such a theory is merely a cloak for our ignorance, it is little more than a restatement of the problem. For it gives us no information as to the real nature of the assumed stimulus, or in what manner it effects its action. The foremost difficulty in the way of a physical or chemical interpretation is the wide range of chemicals and forces all of which are capable of producing artificial parthenogenesis. None of the theories recently advanced seems capable of explaining all or nearly all of the facts. As a result there has been of late again a return to those vague ideas which challenge attack.

In almost all hypotheses of artificial parthenogenesis, the process, as a result of which a membrane is either pushed out or formed "de novo" around the egg, plays an important rôle. This so-called "membrane formation" is readily observed in the egg of the sea-urchin, and it is in *Arbacia* that I have attempted to study it. I am indebted to Prof. T. H. Morgan for the use of a Columbia table in the Marine Biological Laboratory at Woods Hole, Mass., where I spent the summer of 1912.

The form studied was *Arbacia punctulata* (Gray). The nomenclature of the membranes of the sea-urchin egg is somewhat varied, and it will be necessary to decide on a series of terms, which, for the sake of clearness, shall be exclusively

[1] From the Cornell University Laboratory of Embryology.

used. As it emerges from the ovary the unfertilized egg is already surrounded by a broad band of gelatinous material, the chorion. Directly underneath the chorion lies the peripheral surface of the egg. After fertilization, a membrane is clearly seen beneath the chorion and some little distance from the egg; this is the vitelline (or so-called fertilization membrane). The presence of this membrane in the unfertilized egg has often been disputed. Hertwig[1] was of the opinion that a definite preformed membrane exists. Such a condition seemed to Fol[2] to preclude the penetration of the sperm, a fact which he had for the first time observed. Accordingly, he states that the egg is not surrounded by a membrane, but by a "hyaline layer." Upon fertilization, he observes that this layer becomes lifted from the egg and forms the vitelline membrane. Herbst[3] first showed that this "hyaline layer" of Fol was a true membrane in that it possessed rigidity and was distinct from the underlying cytoplasm. He found on pressing unfertilized eggs under a cover glass, that the protoplasm of the egg flowed out, leaving behind the membrane.[4] Schücking[5] isolated the membrane by cutting the eggs. Finally Kite[6] has been able to dissect away the membrane with the aid of a Barber pipette. No doubt the difficulty in observing the membrane is due to the fact that its refractive index is almost identical with that of sea-water. If the refractive index of sea-water is increased, *e. g.*, by the addition of a protein, the vitelline membrane becomes clearly visible. The observation of this membrane in such liquids of greater refractive index has led several observers to suppose that it is "formed" there. Since membranes "formed" in this way are penetrable to sperm, Loeb[7] has proposed to call them "pseudomembranes."

In as much as the presence of a preformed membrane has been demonstrated by a number of observers, it is incorrect to speak of the "formation" of a vitelline membrane at fertilization.

[1] O. Hertwig, *Morph. Jahrb.*, I., 347 (1875).

[2] H. Fol, *Ann. d. sci. phys. et nat.*, LVIII., 439 (1877); *ib.*, *Mem. d. l. Soc. d. phys. et d'hist. nat. d. Genève*, XXVI. (1879).

[3] C. Herbst, *Biol. Centralbl.*, XIII., 14 (1893).

[4] This fact had already been noted by Hertwig (*loc. cit.*).

[5] Schücking, *Arch. f. d. ges. Physiol.*, XCVII., 68 (1903).

[6] G. L. Kite, *Science*, N. S., XXXVI., 562 (1912).

[7] J. Loeb, *Arch. f. Ent. Mech.*, XXVI., 82 (1908).

Thus, although this phrase is in general use, I have discarded it in preference for the more truly descriptive term "elevation." This is in accord with the German practice.

Loeb claims that the process of normal membrane elevation is too rapid for observation. It is necessary to retard the process by cooling to 3° or 4°, when, on fertilization under these conditions, small rounded elevations, the so-called "bubbles" appear on the egg surface; these flow together and their fused outer boundary becomes the vitelline membrane. A similar effect is observed on treatment with butyric acid. I believe that bubble formation is abnormal, and I shall consider its true cause later.

In the fertilized egg the space between the vitelline membrane and the egg is known as the perivitelline space (Membranraum). Some few minutes after fertilization a hyaline layer appears to be differentiated at the external border of the cytoplasm. This is the hyaline layer (ectoplasmic layer, etc.).

The importance of a study of membrane elevation is twofold. In the first place, it may serve as a device for the prevention of polyspermy in normal fertilization, and is, therefore, of physiologic significance. Secondly, in recent years, this process has played an important part in theories of fertilization and artificial parthenogenesis. Robertson[1] goes so far as to confuse membrane elevation with fertilization, considering the two as synonymous.

Although from time to time various ingenious hypotheses have been advanced to explain membrane elevation, the only theory at all plausible was advanced by Fol[2] who was the first ever to consider the problem. Before discussing Fol's view I shall first mention several other explanations which have been suggested. Schücking[3] thought that the preformed membrane was split by the absorption of water, so that a double membrane was produced on fertilization. Such a process would be difficult to explain physically, and no one has ever attempted to do so. Fischer and Ostwald[4] regard membrane elevation as due to the exit from the egg of a fluid which is given off as a result of coagulation of a portion of the protoplasm. The rapidity with

[1] T. B. Robertson, *Arch. f. Entw. Mech.*, XXXV., 64 (1912).

[2] Fol, *Mem. d. l. Soc. d. phys. et d'hist. nat. d. Geneve*, XXVI. (1879).

[3] Loc. cit.

[4] M. Fischer and Wo. Ostwald, *Arch. f. d. ges. Physiol.*, CVI., 229 (1905).

which the membrane is thrust out is but one of the difficulties which such a theory would have to surmount. Traube[1] considers artificial membrane elevation as the result of a secretion after a return to normal sea-water, but in all but a few cases, such a return to sea-water is not necessary. McClendon[2] is of the opinion that an electro-positive (or acid) colloid secretion is[3] poured out from the egg which reacts with the supposedly electro-negative chorion to produce the vitelline membrane. This view is, of course, not in accord with the evidence cited above in favor of the preëxistence of a vitelline membrane. Elder[4] has recently adopted a conclusion identical with that of McClendon, to whom, however, he makes no acknowledgment. He states that the vitelline membrane does not "form" on eggs which lack a chorion. But these eggs are evidently not in good condition, they are probably overripe, and such eggs have been shown by Fol and Loeb incapable of pushing out a vitelline membrane. Elder also states that if the chorion be removed, membrane elevation does not occur (a fact previously claimed by McClendon) but his method of removal involves keeping the eggs 12–14 hours in artificial sea-water. This, of course, precludes membrane elevation. Such evidence is directly controverted by the fact observed by Lyon[5] that after a most vigorous treatment in the centrifuge normal membrane elevation occurs. In eggs treated in this manner the chorion is always absent.

Fol[6] in discussing membrane elevation in *Asterias* eggs, a process which he states is exactly similar to membrane elevation

[1] J. Traube, *Biochem. Zeitsch.*, XVI., 182 (1909).

[2] J. F. McClendon, BIOL. BULL. XXII., 157 (1912).

[3] As proof that this colloid, which is assumed to lie directly beneath the membrane after fertilization, is electro-positive, McClendon states that the passage of an electric current through fertilized eggs in sea-water causes the membrane to "bulge out" towards the cathode. This effect, however, may very well be due to an attempted migration of the egg in the direction of the anode, towards which electrode, most if not all cells migrate (probably because of the alkalinity of protoplasm). The fact that eggs which have ruptured their membranes do not migrate to the anode (McClendon, *Amer. Journ. Physiol.*, XXVII., 273 (1910)) is probably due to a retarding action of the film of water adjoining the glass slide (see W. B. Hardy and H. W. Harvey, *Proc. Roy. Soc.*, B. LXXXIV., 217 (1911)).

[4] J. C. Elder, *Arch. f. Ent.-Mech.*, XXXV., 145 (1912).

[5] E. P. Lyon, *Arch. f. Ent. Mech.*, XXIII., 151 (1907).

[6] Loc. cit.

in the eggs of the sea-urchins, offers the following explanation. A jelly exists between the surface of the egg and the elevated membrane, for the distance between the egg and membrane always remains uniform on all sides. The pushing out of the membrane is due to the absorption of water by this gel. Herbst[1] in a study of membrane elevation in sea-urchin eggs, supports Fol's interpretation. A similar view is likewise expressed by Krassuskaja and Landau[2] and by R. Hertwig.[3] Nevertheless, the view is sometimes regarded as due to Loeb, who first proposed it in 1908. Let us follow the latter's reasoning a little more closely.

In 1900, he regarded membrane elevation as due to coagulation. In 1905,[4] he thought of it as a secretion. In 1907 he began to consider it as due to liquefaction. He first proves that in general the same types of treatment will produce hæmolysis of the red blood cells and the throwing off of a membrane in *Arbacia*. Then he proceeds to adopt the current theory of hæmolytic action to account for the membrane elevation. Köppe and others had attempted to show that the solution or liquefaction of lipoids was necessary for hæmolysis. Accordingly, Loeb[5] expresses the view that membrane elevation is primarily due to a solution of lipoids at the periphery. This view was also taken up in Loeb's laboratory by v. Knaffl-Lenz,[6] who brought forth evidence that the lipoid in question was lecithin. But the actual force which produced the membrane elevation Loeb believes to be due to the swelling of a colloid. He conceives of this colloid as being given off at the time of fertilization and immediately swelling and thus distending the membrane. This does not accord with his previous statements that the unfertilized egg is naked, but Loeb surmounts this difficulty by assuming an "Oberflächenlamelle" or a differentiated surface layer. In 1909, Loeb[7] is no longer certain that solution of lipoids is necessary,

[1] C. Herbst, loc. cit.

[2] Krassuskaja and Landau, *Biol. Centralbl.*, XXIII., 613 (1903).

[3] R. Hertwig in O. Hertwig's *Handbuch der Entwicklungslehre der Wirbeltiere*, I. (1), p. 484.

[4] Univ. Cal. Publ. Physiology, II., 123 (1905).

[5] Loeb, *Arch. f. d. ges. Physiol.*, CXX., 196 (1908).

[6] v. Knaffl-Lenz, *Arch. f. d. ges. Physiol.*, CXXIII., 279 (1908).

[7] Loeb, "Chem. Entwicklungserregung," p. 248.

but he still clings to the view that the swelling of a colloid is involved. This is the identical view earlier expressed by Fol, Herbst, etc. Because of the similarity between membrane elevation and hæmolysis, Loeb introduces the term lysine to denote any substance of unknown chemical composition which will produce membrane elevation and cytolysis. It is evident that the sperm may contain a lysine.

R. S. Lillie[1] suggests a possible cause of swelling. He assumes that at fertilization the outer surface layer of the egg becomes more permeable to the salts of sea-water. These can now exert no osmotic pressure against it, but since this outer film or membrane must still remain impermeable to the colloids within, these do exert osmotic pressure,[2] and an inflow of water with consequent swelling of some of the peripheral cytoplasm occurs. The cause of such an increase in permeability has only been hinted at. Its actual occurrence Lillie bases on the evidence of McClendon, Lyon and Shackell, and Harvey. But even granted such a chang of permeability, might it not just as wel be regarded as a result rather than as a cause of membrane elevation? Harvey's[4] view is very much the same. He agrees with Loeb that the membrane elevation is due to the swelling of a colloid, and at the same time brings evidence in favor of Lillie's hypothesis of increased permeability of plasma membrane. But Harvey also believes that the presence of the "membrane substance" is

[1] R. S. Lillie, *Amer. Journ. Physiol.*, XXVII., 301 (1911).

[2] Lillie considers osmotic pressure and "Quellungsdruck" synonymous, but this is obviously improper, for the osmotic pressure of colloids is almost negligible, the "Quellungsdruck," on the other hand, may exert a pressure of over 40 atmospheres.

[3] J. F. McClendon, *Amer. Journ. Physiol.*, XXVII., 240 (1910); Lyon and Shackell, *Science*, N. S., XXXII., 249 (1910); Harvey, *Science*, N. S., XXXII., 565 (1910). The first two papers do not prove increased permeability of the membrane. They only show that fertilized eggs stain more readily than those not fertilized, a phenomenon which may also depend upon an increased rate of adsorption after fertilization, or to a lowering of osmotic pressure, such as Bachmann (*Arch. f. d. ges. Physiol.*, CXLVIII., 141 (1912)) concludes for the egg of *Triton*. McClendon's results are also unsatisfactory. He observed an increased conductivity after fertilization, and from this concluded an increased permeability of the outer layer or membrane. Such an increase in conductivity may well have been caused by a disintegration of some of the eggs. The action of the electric current, or the centrifuging which preceded conductivity determinations, might readily produce disintegration.

[4] E. N. Harvey, *Journ. Exp. Zoöl.*, VIII., 355 (1910).

due to a reaction or reactions brought about by the escape of $CO_2$ as a result of increased permeability. Unfortunately, however, he does not bring up any evidence in favor of this accessory hypothesis, and it is, indeed, difficult to understand how both reaction and swelling can take place in so short a time.

The appended list is an enumeration of all the various methods by which membrane elevation may be artificially induced in sea-urchin eggs. Sometimes artificial parthenogenesis has been described without any records to show if membrane elevation occurred. Several of these methods are included in the list, where they are followed by a question mark. Most acids only cause membrane elevation after the eggs have been restored to normal sea-water, but in all other cases the process occurs directly.

So-called lipoid solvents:
- Chloroform (Hertwig).
- Toluol, benzol, xylol, oil of cloves, creosote (Herbst).
- Ether, alcohol (Matthews).
- Amylene, phenol (Loeb).

Distilled water (Schücking).
Dilution of sea-water (Schücking).
Isotonic NaCl, KCl (Lillie).
Soap (Loeb).
Saponin, digitalin, solanin (Loeb).
Bile salts (v. Knaffl-Lenz).
Sea-water charged with $CO_2$ (Delage, Lyon).
Passage of hydrogen and oxygen (Matthews).
Shaking (?) (McClendon).
Heat (34° Loeb) (32° (?) McClendon).
Alkalis (Loeb, Schücking).
KCN (?).
Metallic copper or silver (Herbst).
Electric current (?) (Schücking).
Blood serum (Loeb).
Oöcytin (Robertson).
Higher fatty acids (Loeb).

Lower fatty acids (Loeb),
Hydroxi-acids (Loeb),
$HNO_3$ and HCl (Loeb),
} Membrane elevation only after return to sea-water.

I shall now attempt to prove that every known method of producing membrane elevation results in a lowering of the surface tension of the liquid surrounding the egg. Proceeding in order, the first class of substances noted are those often grouped as

lipoid solvents. The surface tension of some of these substances is listed in Landolt and Börnstein, "Physicalische Chemische Tabellen."

| Substance. | Temperature. | Surface Tension (Dynes per Cm.) |
|---|---|---|
| Chloroform | 20° | 26.72 |
| Toluol | 17.5° | 28.52 |
| Benzol | 20° | 30.2 |
| Xylol | 15.7° | 28.97 |
| Ether | 20° | 16.49 |
| Alcohol | 20° | 22.03 |
| Amylene[1] | 16.5° | 17.21 |
| Phenol | 36.5° | 41.3 |

It will be noticed that the first four of these substances are practically insoluble in sea-water. Herbst found that chloroform and toluol must be shaken with sea-water to produce the necessary effect.

*Distilled Water and Dilution of Sea-water.*—The surface tension of distilled water is 75 dynes per centimeter. The addition of inorganic salts always increases the surface tension gradually in proportion to the concentration of each salt present, and, by the work of Valson, Röntgen and Schneider, Whatmough,[2] etc., we should estimate the surface tension of sea-water at approximately 77 dynes per centimeter. Hence diluted sea-water and distilled water have a somewhat lower surface tension than sea-water, and it is to this fact that I attribute their action in causing membrane elevation.

*Isotonic Salt Solutions.*—Lillie[3] first showed that pure isotonic (*i. e.*, 55M) solutions of sodium salts caused membrane elevation, the order of effectiveness of anions being that of the lyotropic series, $Cl > Br > ClO_3 > NO_3 > CNS > I$. The effect of these salts was inhibited by $CaCl_2$ and $MgCl_2$. It was shown by Whatmough[4] and others that equivalent normal solutions of

[1] The surface tension of amylene is not given in Landolt and Börnstein, so I have taken the value given in Castell-Evans' Physico-Chemical Tables. Creosote is not included in the list as it is a mixture of various substances; most of these, however, have a lower surface tension than water.

[2] Valson, *Ann. de chem. et de phys.* (4), XX., 361 (1870); Röntgen and Schneider, *Wied. Ann.*, XXIX., 165 (1886); Whatmough, *Zeit. f. phys. Chem.*, XXXIX., 129 (1902).

[3] R. S. Lillie, *Amer. Journ. Physiol.*, XXVI., 106 (1910).

[4] *Loc. cit.*

chlorides raise the surface tension of water by approximately equal amounts. Hence a solution of a bivalent salt (such as $MgCl_2$ or $CaCl_2$), which is equal in molecular concentration to a solution of NaCl, gives an increase of surface tension which is about double that produced by the NaCl. Sea-water, consisting as it does of monovalent and bivalent salts, has a higher surface tension than an isotonic solution of sodium chloride (55M). It also contains sulphates which raise surface tension more than monovalent chlorides. The fact, therefore, that isotonic solutions of NaCl, etc., do cause membrane elevation is exactly in accord with my view. Moreover, the addition of $CaCl_2$ and $MgCl_2$, two bivalent salts, raises the surface tension, and, hence, as might be expected, membrane elevation no longer takes place. Finally, the additional evidence of the correctness of my interpretation is furnished by the order of effectiveness of the ions: $Cl < Br < ClO_3 < NO_3 < CNS < I$. For we know the surface tension increasing powers of the anions to be $Cl > Br > NO_3 > I$.[1]

*Soap.*—Na oleate greatly lowers the surface tension of water[2] and Loeb[3] finds that when it is added to M/2 NaCl it increases the membrane elevating power of the solution. The fact that as much as 2 per cent. of Na oleate is necessary is probably due to the tendency of the NaCl present to salt it out of solution.

*Saponin.*—Freundlich[4] determines the surface tension of a solution of saponin as 52 dynes per cm. Probably solanin and digitalin also lower surface tension.

*Bile Salts.*—These substances are extremely effective in lowering surface tension. For example, Lewis[5] gives the surface tension of a 0.2 per cent. solution of sodium glycocholate as 44.98 dynes per centimeter (at 14°).

*Sea-water Charged with $CO_2$.*—The surface tension of gases is zero, hence we should expect them to lower the surface tension of water when dissolved in it. This assumption was proven

[1] Röntgen and Schneider, *Annalen der Physik u. Chem.*, XXIX., 209 (1886); Traube, *Journ. Prakt. Chem.*, CXXXIX., 177 (1885).

[2] Donnan, *Zeits. f. phys. Chem.*, XXXI., 42 (1899).

[3] Loeb, "Chem. Entwicklungserregung," 1909, p. 140.

[4] "Kapillarchemie," p. 56.

[5] Lewis, *Zeit. f. physik. Chem.*, LXXIV., 619 (1910).

correct by Bellati and Lusanna[1] and more recently by Bönicke,[2] who finds that ten volumes per cent. of $CO_2$ lower the surface tension of water by about one dyne.

*Passage of Hydrogen and Oxygen.*—Matthews[3] found that if hydrogen be passed through sea-water containing eggs for ten minutes, then oxygen for ten minutes, and once more hydrogen for ten minutes, the eggs thus treated begin to develop. He does not state whether membrane elevation occurs, but we can readily understand that it may well do so, for both hydrogen and oxygen, no doubt, lower the surface tension.[4] As Matthews says he ran the hydrogen through swiftly, the impurities (*e. g.*, $SO_3$, $AsH_3$) could not have been completely removed, and probably, for this reason, continued exposure to hydrogen gas was found injurious.

*Shaking.*—Matthews[5] finds that shaking will cause artificial parthogenesis in starfish eggs, but met with no success in his experiments in *Arbacia*. However, McClendon reports that segmentation occurred after shaking in a vial for five minutes. He does not state if membrane elevation occurred, but from a chance experiment of my own, I believe that it may do so. Vigorous shaking would produce a lowering of surface tension in that it would increase the amount of air absorbed (for effect of gases see above).

*Heat (to 32° or Over).*—As is well known, a rise of temperature produces a lowering of surface tension in all cases. McClendon states that exposure to 32° for four minutes is sufficient to produce segmentation, but he does not state if membrane elevation occurred.

*Alkalis.*—Upon the addition of NaOH, KOH, or $Na_2CO_3$, to sea-water, there is an immediate precipitation of magnesium hydroxide. This fact has never been noted by Loeb, McClendon, or Schücking, all of whom have used alkaline sea-water. It is,

[1] Bellati and Lusanna, *Atti. 1st Venet.* (6), VII. (1889); ref. in *Wied. Beibl.*, XIV., 18 (1889).

[2] K. Bönicke, Dissert., Münster (1905).

[3] A. P. Matthews, *Amer. Journ. Physiol.*, IV., 343, 1901.

[4] The latter gas was investigated by Bönicke, who found that it lowered surface tension.

[5] A. P. Matthews, *Amer. Journ. Physiol.*, VI., 142 (1902).

however, a necessary consequence of the fact that sea-water contains salts of magnesium (which constitute nearly 1/6 of the total salt content). Magnesium salts are always precipitated in the presence of NaOH or KOH, this reaction often being employed in qualitative analysis. As a result of this precipitation, the salts which tend most to raise the surface tension (see p. 350) are precipitated and the surface tension of the sea-water decreases slightly. As might be expected, alkalis are not very powerful agents in producing membrane elevation.

*Blood Serum.*—Loeb[1] first showed that the body fluid of certain annelids of the family Sipunculidæ was capable of producing membrane elevation even in considerable dilution. As I know nothing of the chemical nature of this fluid, it is useless for me to consider this case. Soon after, he found that mammalian blood serum was also effective.[2] 6.5 volumes of the serum are mixed with one volume of 2.5 M NaCl and the resultant solution is then diluted with 1–9 parts of sea-water. Loeb's results show that the per cent. of eggs which "form a membrane" as a result of treatment with this solution is small, in the majority of cases not a single egg throws off its membrane. In order to obtain a higher percentage of membrane elevation, sensitization must be resorted to. This may be effected either by heating (to 31°–34°) or by the addition to the serum of 3/8 M $BaCl_2$ or $SrCl_2$.

First I shall consider the effects of sensitization, as I believe that this process is in itself capable of producing membrane elevation. The result of heating has already been considered, it having been noted that a lowering of surface tension results. As for the addition of $BaCl_2$ or $SrCl_2$, the result must be obvious. Either one of these salts causes an immediate precipitation of sulphates. Loeb in fact mentions a precipitate of $BaSO_4$ in the case of $BaCl_2$, but fails to do so in the case of $SrCl_2$. Such a precipitation has two effects. (1) The strontium chloride added is precipitated from the solution, which then becomes more dilute. (2) The sulphates of sea-water are replaced by chlorides which are somewhat less effective in elevating the surface tension (see p. 351). Thus sensitization is not such a mysterious

[1] J. Loeb, *Arch. f. d. ges. Physiol.*, CXVIII. (1907).

[2] J. Loeb, *Arch. f. d. ges. Physiol.*, CXXII., 196 (1908); CXXIV., 37 (1908).

process as Moore[1] and Robertson[2] would have us believe when they suppose that it is similar to the process of mordanting before dyeing. An experiment of Moore's seems to show that my explanation is correct. Upon placing eggs in 3/8 $SrCl_2$, and then removing directly into sea-water, sensitization occurs, but if the eggs are first washed in NaCl before being placed in sea-water, the effect of the $SrCl_2$ disappears. Evidently the washing away of the $SrCl_2$ prevents its reaction with sea-water.

The effect of serum alone is very slight. Of nine samples of ox-sera tried by Robertson, only one was effective upon sensitized eggs. I attribute the effect of blood serum to two causes. In the first place, before dilution with sea-water the salt content of the "isotonic" serum is almost wholly NaCl, together with a small amount of KCl. The effect of isotonic NaCl and KCl solutions has already been discussed. Secondly, blood serum contains considerable amounts both of oxygen and of carbon dioxide. The total volume of these gases contained in blood varies greatly. On the average, arterial blood possesses about 20 vols. per cent. of oxygen and 40 vols. per cent. of carbon dioxide, whereas venous blood contains approximately 7 volumes per cent. of oxygen and 50 volumes per cent. of carbon dioxide.[3] Blood serum is richer in carbon dioxide than the blood itself.[4] Thus the serum used by Loeb to produce membrane elevation must contain from 5 to 25 volumes per cent. of carbon dioxide in addition to a lesser amount of oxygen.

No doubt some of this $CO_2$ is loosely combined, but even in this case it would exert a dissociation pressure which would increase the amount present in solution. Using ox-blood obtained from a slaughter-house, Robertson found a difference in the serum obtained from dark and that obtained from light blood, the former being in all cases more effective. He is at a loss for an explanation, but suggests that the dark blood was obtained from animals deprived of water for some time. Probably the dark color of the more effective blood is associated with the presence of a higher per cent. of $CO_2$. The action of $CO_2$

[1] A. R. Moore, Univ. of Cal. Pub'. (Physiology), IV., 91 (1912).

[2] T. B. Robertson, *loc. cit.*, p. 345.

[3] See Oppenheimer's "Handbuch der Biochemie," IV. (1).

[4] Hammersten, "Physiological Chemistry," 6th Amer. edition, p. 804.

(and oxygen) in lowering surface tension has already been discussed.

KCN.—Dilute potassium cyanide is practically equivalent to KOH + HCN, as it undergoes hydrolysis. Hence, the statements in regard to alkalis apply also here.

*Metallic Copper or Silver.*—Herbst[1] found that membrane elevation was induced by the presence of metallic Cu or Ag. His results were confirmed by Matthews,[2] whose experiments however were performed on starfish eggs. Herbst used silver reduced from silver nitrate, and also a silver coin. In the former case, silver nitrate is probably present and effects a precipitation of chlorides with a consequent dilution of the sea-water and an exchange of chlorides in solution to nitrates. (For a consideration of the effects of dilution, and of the relative effects of various ions, see p. 350.) In the latter case, it is necessary to note that all silver coins are alloys of copper, a metal which Matthews finds to be much more potent than the silver coin itself.[3] The action of copper is due to the fact that this metal is attacked by NaCl in the presence of air. As a result of this reaction copper oxychloride is formed and the solution becomes alkaline.[4] Thus the action of copper is a special case of the action of alkalis, and the metal does not produce an "action at a distance," as Matthews supposes.

*Electric Current.*—Although an electric current has been used by Schücking and McClendon, neither of these authors states whether membrane elevation occurs. In both cases, copper wires were probably used, so that membrane elevation may have been induced by the action of copper itself, as shown above.

*Oöcylin.*—This is an hypothetical substance isolated by T. B. Robertson[5] from blood serum and from sperm. Although called a "fertilizing agent" it produces normal membrane elevation on "sensitized" eggs only. On "unsensitized" eggs it produces

[1] C. Herbst, *Mitth. a. d. zool. Station z. Neapel*, XVI., 445 (1904).

[2] A. P. Matthews, *Amer. Journ. Physiol.*, XVIII., 39 (1907).

[3] Metallic silver may itself react for NaCl in the presence of oxygen changes it into AgCl with the simultaneous formation of NaOH. (See Gmelin-Kraut, *Lehrbuch der anorg. Chemie*, II. (2), p. 26.) This reaction probably takes place very slowly, however.

[4] Tilden, *Soc. Chem. Ind.*, V., 84 (1886).

[5] T. B. Robertson, *Arch. f. Ent. Mech.*, XXXV., 64 (1912).

agglutination, and sometimes "blister" formation. Its power of producing membrane elevation is, therefore, chiefly due to the process of "sensitization," the real nature of which has already been considered. The agglutinating effect is due to the presence of free hydrochloric acid. Robertson prepares oöcytin by precipitating blood serum with $BaCl_2$ dissolving the precipitate in n/10 HCl, reprecipitating with acetone, and then redissolving in n/10 HCl. The result of this process can not be other than the isolation of a protein combined with HCl, for we know both $BaCl_2$ and acetone as protein precipitants.[1] And, although Robertson exactly neutralizes his final product with NaOH, he, of course, does not neutralize the combined acid which has no action on the color of the indicator. On dilution, the combined acid is split off. Thus Robertson finds that a dilution of one part in two hundred is necessary in order that the oöcytin become effective. Dilute HCl will produce agglutination in the same way as oöcytin.

*Higher Fatty Acids.*—Loeb[2] finds that very dilute solutions of heptoic, octoic, nonoic, and caproic acids produce membrane elevation in eggs exposed to them. As Forch[3] and others have shown, the higher fatty acids lower surface tension markedly, even when present in great dilution. The readiness with which a fatty acid lowers surface tension is found to be in direct relation to the number of carbon atoms it contains.

*Lower Fatty Acids. Mineral Acids.*—There remain to be considered only those cases in which membrane elevation occurs after a return to sea-water. In all of these cases, the solutions in which the eggs are first placed are acidified. All of the acids used lower surface tension and their effectiveness is in direct measure to the readiness with which they do so. The lower fatty acids, formic, acetic, propionic, butyric, and caproic, lower surface tension markedly in solution, and this power increases as we ascend in the series.[4] Similarly, the effectiveness in producing membrane elevation increases with the number of carbon atoms.

[1] Mann ("Physiological Histology," p. 102) finds acetone a precipitant of serum globulin and other proteins.

[2] Loeb, "Chemische Entwicklungserregung," 1909, p. 110.

[3] Forch, *Wied. Ann.*, LXVIII., 801 (1898).

[4] Forch, *loc. cit.*

Dibasic acids, such as oxalic, succinic, and tartaric, do not lower surface tension nearly so much[1] and they have comparatively little effect upon sea-urchin eggs. Hydroxi-acids act in a fashion similar to the dibasic acids. Finally, hydrochloric and nitric acids have been found to be least effective.[2] These acids lower the surface tension but slightly, as has been shown, for example, by Röntgen and Schneider.[3] Loeb[4] could obtain no results with sulphuric acid, and it is interesting to note that this acid does not produce a lowering of surface tension when dilute. The question arises as to the reason that a return to sea-water is necessary in the case of the above mentioned acids. This point will be made clear later.

I have now shown that every known method of producing membrane elevation results in a lowering of surface tension. I also found that a number of hitherto untried substances which lower surface tension, can also be used to produce membrane elevation.

*Acetone.*—According to von Knaffl-Lenz's view that it is the liquefaction of lecithin which causes membrane elevation (see p. 8, l. 12), acetone should be entirely ineffective, as it is well known for its power of precipitating lecithin. On the contrary, I found acetone a very convenient means of producing membrane elevation. Eggs placed in a solution of 3 or 4 c.c. acetone + 25 c.c. of sea-water push out membranes, and cytolysis follows. A very rapid action results if the eggs are placed in more concentrated solutions of acetone.[5] This can be observed by dropping some eggs into a drop of acetone under the microscope.

*Chloretone.*[6]—This substance is very effective in producing a lowering of surface tension. I found that a 0.1 per cent. solu-

[1] J. Traube, *Liebig's Annalen*, CCLXV., 27.

[2] In 1905, Loeb (Univ. Cal. Publ. Physiology, II., 113) obtained "practically negative" results on adding these acids to sea-water. In 1909 (*Biochem. Zeitsch.*, XV., 254), he made up his solution in *M*/2 NaCl, which in itself has been shown to cause membrane elevation, and in this way he met with occasional success.

[3] *Loc. cit.*

[4] Loeb, "Chemische Entwicklungserregung," p. 105.

[5] Solutions of acetone in sea-water are not as concentrated as they seem, for the acetone tends to be salted out of solution. (See Bernthsen, "Organische Chemie," p. 170.) The surface tension of acetone at 16.8° is 23.35 dynes per centimeter.

[6] Chloretone is a trade name for tri-chlor tertiary butyl alcohol.

tion lowered the surface tension of water by almost one fourth of its value. As was expected, therefore, membrane elevation was produced when a few crystals of chloretone were added to sea-water containing eggs.

*Urethane.*—According to an approximate determination, a 0.5 M solution of urethane has a surface tension of about 61.3 dynes per centimeter, a value considerably below that of pure water. Hence all solutions of urethane produce membrane elevation (and cytolysis) whether they be isotonic, hypotonic, or hypertonic. Solutions of urethane in sea-water likewise give results, although this substance is not as effective as chloretone.

*Chloral Hydrate* acts in similar fashion to chloretone and urethane.

*Esters.*—All esters possess the property of lowering surface tension.[1] The following esters were used and proved effective in producing membrane elevation. Methyl acetate, ethyl acetate, ethyl butyrate, methyl salicylate.

The substances which lower surface tension are very numerous Not all, however, produce membrane elevation. In general, there are three classes of exceptions.

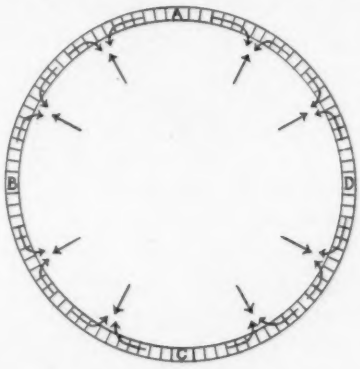
1. *Colloids.*—Many proteins lower surface tension but do not cause membrane elevation.

2. *Protein Coagulants.*—If the solution is strongly coagulative, no sign of membrane elevation will appear. However, if the coagulative action is not very strong, or if the surface tension is quite low, membranes are often pushed out. Under these conditions, a number of little elevations, the so-called "bubbles" or "blisters" are often seen to surround the egg. Loeb and his pupils consider these "blisters" as a preliminary stage in membrane elevation, but they can be observed only when a protein coagulant is present. By adding protein coagulants and at the same time lowering the surface tension, "blister" formation can always be induced. For example, if the eggs are subjected to a solution of acetamide, which contains free acetic acid, they immediately become surrounded with blisters. Similar results are gained with aqueous solutions of picric acid. If the surface tension is low enough, membranes sometimes are formed.

[1] Cf. J. Traube, *Ber. d. deutsch. chem. Gesellsch.*, XVII., 2294 (1884).

3. *Sugar and Glycerin.*—A solution of cane sugar possesses a surface tension very slightly above that of pure water,[1] a glycerin solution has a surface tension below that of sea-water. Thus, both of these solutions have a lower surface tension than sea-water, but if eggs are placed in them, no membrane elevation occurs. The reason for this exception will be considered shortly.

I have shown that membrane elevation depends upon a lowering of surface tension, and I shall now consider the mechanism of the process. As has already been pointed out, the unfertilized egg is surrounded by a membrane, which is probably a gel or semi-gel. In the appended diagram, *ABCD* represents this gel,




whose parts pull on each other by reason of their surface tension. As a result of this pull, the underlying egg contents, and more especially the peripheral portions of the egg, are under pressure. This pressure inward is compensated by a "Quellungsdruck" due to a tendency of the proteins to swell. Upon a diminution of surface tension, the pressure inward is relaxed and the protein or proteins directly beneath the membrane immediately swell, thus pushing out the membrane. If the surface tension remains lowered, the entire egg swells, and cytolysis results.

It was found that not all lowerings of surface tension produced membrane elevation, there being three types of exceptions. The reason for these exceptions can now be explained. Although many colloids lower surface tension, they are unable to diffuse

[1] Forch, *loc. cit.*

into the membrane, and thus lower its surface tension. Moreover, because of their inability to penetrate the membrane, they exert pressure against it, osmotic or otherwise. Secondly, coagulative agents prevent membrane elevation, since they make it impossible for the protein which causes the phenomenon to swell. Probably the main reason that glycerin and sugar solutions do not produce membrane elevation is their action in increasing the viscosity of the gels.[1] When the viscosity of the membrane is augmented, it loses its fluidity and can no longer be pushed out. This hardening effect can be observed directly by placing fertilized eggs with membranes in a sugar solution and examining the result under the microscope.

When acids are used to produce membrane elevation, a return to sea-water is usually necessary. No doubt, the acid by virtue of its coagulative power inhibits membrane elevation. On a return to sea-water the coagulative effect is lost by dilution, but enough acid has probably been adsorbed by the membrane to lower its surface tension sufficiently for membrane elevation.

For the sake of simplicity, one of the factors which must have an influence on membrane elevation has been omitted from the discussion. It has been shown[2] that in the presence of chlorides, bromides or nitrates, gelatine shows a greater tendency to swell than in pure water, but that sulphates, sugar, and glycerin have a retarding effect. If membrane elevation is the result of the swelling of a colloid, and if this colloid behaves as gelatine, the presence of chlorides, bromides, and nitrates would accelerate the process, whereas sugar and glycerin would retard or prevent it. The effect of these substances is probably of secondary importance.

In the course of the argument, evidence has been adduced which may also serve to explain other biological problems. Although the toxicity of distilled water has often been noted, no one has ever offered a satisfactory explanation. Bullot[3] found that the purest water obtainable (redistilled in platinum and quartz) was toxic to the fresh water *Gammarus*. It is believed that this

[1] Leick, *Drude's Annalen*, XIV., 139 (1904).
[2] See Freundlich, "Kapillarchemie," p. 512.
[3] G. Bullot, Univ. Cal. Pub. Physiol., I., 199 (1904).

toxicity is due to the lower surface tension possessed by pure water (see p. 350). The experiments of R. Lillie have often been adduced as evidence in favor of the "theory of antagonism." Lillie's results find a most suitable explanation when the surface tension of the solutions is considered (see p. 351). A recent paper by Lillie[1] seeks to show an antagonism between salts and anæsthetics in their action on starfish and sea-urchin eggs. This would seem to contradict the results gained in this paper. All that Lillie shows, however, is that various anæsthetics, such as ether, chloroform, etc., exert a slight protective influence on eggs partially cytolyzed with isotonic NaCl solution. This protective action is probably due to the effects of the anæsthetics upon the bacteria which are always found to infest cytolyzing eggs. When these are killed the egg is able to live longer. Gorham and Tower[2] showed that sea-urchin eggs could be kept in healthy condition in sterile sea-water for 11 days or longer, whereas they soon disintegrate if exposed to bacteria.

## Summary.

1. All known methods of producing an elevation (formation) of the vitelline membrane in the egg of the sea-urchin result in a lowering of surface tension.

2. The following substances, all of which lower surface tension, were also found effective in producing membrane elevation: acetone, chloretone, urethane, chloral hydrate, methyl acetate, ethyl butyrate, methyl salicylate, acetamide, picric acid.

3. A simple physical explanation of the process is given, which is based on Fol's original interpretation.

I desire to express my sincere thanks to Prof. B. F. Kingsbury of Cornell University for helpful advice in the preparation of this paper. Thanks are also due to Prof. W. D. Bancroft, who has been consulted on several questions of a chemical nature.

[1] R. S. Lillie, *Amer. Journ. Physiol.*, XXX., 1 (1912).

[2] F. P. Gorham and R. W. Tower, *Amer. Journ. Physiol.*, VIII., 175 (1902).

# EXPERIMENTS ON COÖRDINATION AND RIGHTING IN THE STARFISH.

LEON J. COLE.

In another paper (Cole, '13) I have presented and discussed the results of a series of experiments on the direction of locomotion of the starfish, *Asterias forbesi*, with respect to morphological axes, in the absence of directive stimuli. The experiments described in the present note were incidental to the more complete series just mentioned. They were performed tentatively to test the feasibility of certain lines of investigation which suggested themselves in the course of the other work, but which I have been unable to prosecute further. While they are not extensive enough to serve as a basis for far-reaching conclusions, they nevertheless supplement to some extent the observations of others, and, it is believed, suggest certain lines of research which might be followed up with profit. All of the experiments here described were made in November, 1909, in the animal behavior laboratory of the department of zoölogy, Sheffield Scientific School of Yale University.

In the locomotion of the starfish it was found that the "unified impulse" was an important factor, and Jennings ('07) had previously determined the same to be true in the righting reactions of the West Coast starfish, *Asterias forreri*, which he studied. The establishing of a "unified impulse" toward performing a definite action, such as turning on certain rays, or crawling in a certain direction, implies, of course, coördination. Loeb ('00) in his book on the "Physiology of the Brain" lays stress on coordination in the starfish, though apparently merely as an inhibiting influence, which prevents the rays not helping in a given reaction from working against its consummation, rather than one which causes them to join actively in bringing it about, that is, by taking part in a "unified impulse" toward that end. In discussing, for example, an experiment which will be mentioned later, he says (p. 63): "The experiments seem to indicate that

in a normal starfish the stimulus produced by the pulling of two or three arms in the same direction has an inhibitory effect on the other arms." Again (p. 65), he explains the movement of a starfish in a direction away from a ray which has been stimulated by saying that "the feet of this arm are drawn in and the arm becomes inactive," and "therefore, according to the parallelogram of forces, a movement away from the point of stimulation will take place." While stimulation of an arm may cause temporary inactivity of the feet of that arm and this might aid in initiating movement in the opposite direction (Moore, '10), the careful observations of Jennings ('07, p. 97) seem, however, to show that this explanation is only partial. He remarks on this point: "It is possible that in some cases when one ray is stimulated locomotion takes place entirely with the other rays, but such cases are very rare; though I have watched carefully for this, I have never seen one. As a rule the walking away from the stimulated region is due, like the usual locomotion of the starfish, to the coöperation and coördination of the tube feet of all the rays . . . . The active tube feet of all the rays are pushed forward in the direction in which the starfish is going; their suckers attach themselves, and by the contraction of the tube feet . . . the starfish is carried forward, the action of all the tube feet aiding in this." My own observations agree in this respect with those of Jennings. It is therefore evident that in locomotion as in righting an impulse is established, by which the different parts act in more or less complete harmony toward the accomplishment of a certain result. It is generally assumed, and is undoubtedly true, that this unity of action is made possible by the mediation of the nervous system.

### Section of Radial Nerves.

The question naturally arises, however, whether it is not possible that an impulse toward the accomplishment of a definite end might be established without nervous connection between the different parts of the organism, just as an earthworm continues its coördinated locomotor contractions after section of the ventral nerve cord, or indeed if the worm itself be cut entirely through and the halves united by thread (Friedländer, 1888).

Here the muscular pull of the forward part acts as the stimulus for contraction in the posterior piece. Loeb's experiment in this connection was negative. He cut the nerve ring of a starfish at two points, nearly opposite to each other, thus severing the nervous connection of certain arms with the others. He found that whereas "the normal starfish requires but a few minutes to turn over, . . . the specimen [operated upon as described] remained on its back the whole afternoon, although the arms were struggling constantly to right it" (Loeb, '00, p. 63). Romanes ('85, p. 296) had, however, previously obtained different results in his experiments on the common British starfish. He states that when animals with the radial nerves severed at the bases of the arms are inverted, "the power of effecting the righting manœuvre is seen to be gravely impaired, although eventually success is always achieved." My experiments on *Asterias forbesi* agree in this respect with those of Romanes.

*Experiment 1.* (Specimen No. 10 of earlier paper.)—In three preliminary trials this specimen required 6 minutes, 5 minutes and 6 minutes respectively for righting, and turned on arms *cd*,[1] *bc* and *cd* in the successive trials. An incision was now made at the base of each arm, thus severing the radial nerves (and of course the radial water canals as well) close to their origin from the circumoral ring. The specimen was again placed on its back in the water, and the various arms at once began to make individual and apparently random movements toward righting.[2] While most of the arms went through various activities, bending and twisting, attaching and pulling, only to let go again, arm *c* persisted only in bending up orally, and did not twist and attach. This resulted finally, at the end of 20 minutes, in its becoming bent over far enough to obtain a hold between *a* and *e*, as shown in Fig. 1*A*. Arms *b* and *d* were also thrown well over at the

[1] Following Jennings ('07) the arms are designated *a*, *b*, *c*, *d* and *e*, beginning at the ray to the right of the madreporite and going around clockwise (cf. Cole, '13, p. 2). Dr. R. T. Jackson has emphasized (*in litt.*) the desirabilty of students of animal behavior, as well as specialists, using for the starfish the nomenclature introduced by Lovèn for the ambulacral and inter-ambulcral areas of Echini. With this view I am in accord and should have adopted the method had it not been that my earlier paper necessitated so much direct comparison with that of Jennings, and a different nomenclature would have made such comparison difficult.

[2] If there is any shock from the operation, it is so slight as to be hardly noticeable.

same time, but were not attached. Soon these latter straightened out again and the attachment of *c* was loosened. Twenty minutes later *c* and *d* bent over together and succeeded in obtaining a hold between *b* and *a* (Fig. 1*B*). By their concerted effort they succeeded in pulling the body over, *e* crossed over *a* (Fig. 1*C*), and at the end of 44 minutes from the time it was placed on its back the starfish had completely righted itself, turning, as seen, on arms *a* and *b*. It now remained quiet for two or three minutes, and then began crawling with *b* in advance, the movement being, however, very slow, as if there were not complete coördination of all the arms.

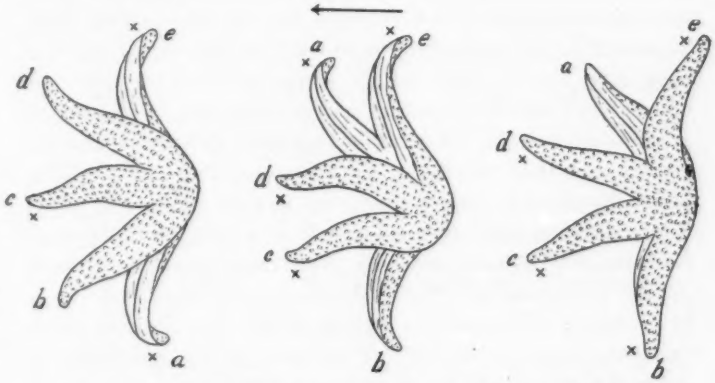



FIG. 1. Diagrams of righting movements of a starfish having the radial nerves severed at the bases of all the arms. Arms attached to substratum are indicated by ×. Arrow shows direction of turning.

*Experiment 2.* (Specimen No. 14.)—In eleven preliminary trials this animal righted itself in from one to five minutes, the average being 2.6 minutes. In ten of the trials it turned on arms *a* + *e*. An incision was now made on the oral side at the base of each arm, severing not only the radial nerve and water canal, but also the muscles and other internal organs, and leaving only the aboral arch of the external skeleton intact. It was now placed on its back in the water. It is unnecessary to give in detail the movements of the arms; it is sufficient to say that they at once began active but entirely uncoördinated movements.

Nevertheless, at the end of 56 minutes the animal had succeeded in righting itself, though arm *b* was still crossed over arm *a*. At the end of one hour, however, from the time it was placed on its back, it had straightened out completely. The turning was accomplished with arms *e* and *a*, although *c* swung over between them and really seemed most instrumental in pulling the animal over.

In both of these experiments the lack of coördination was very apparent, as mentioned by both Romanes and Loeb. Neither could it be determined that in either experiment a definite impulse toward turning in a given direction was established, as is normally the case. In fact, especially in the second experiment, it was noticed several times that as two or more arms happened at the moment to be working in concert, the righting could have been effected easily if another arm had but ceased its efforts in an opposite direction, to say nothing of helping in the same direction. The final turning appeared to be nothing but a chance occurrence when enough arms happened to be pulling in one direction to turn the specimen in spite of its other arms. Thus, although there resulted complete lack of coördination, as Loeb states is the case when the nerve connections are severed, the end result was nevertheless finally accomplished, apparently by accident. The pull of one arm on another does not seem, therefore, to be a sufficient stimulus to induce a definite coordination of the tube feet in the second arm.

The second specimen did not crawl after righting itself. It would be interesting to test whether by exerting a steady pull in one direction on such an "uncoördinated" specimen, a more or less unified impulse to crawl in that direction might not be induced, comparable to the crawling of the severed earthworm.[1] This experiment was not tried, but the lack of coördination shown in the righting would make it seem that a negative result might in all probability be expected.

[1] These experiments might be made even more comparable by severing the rays of the starfish completely from the disc and then sewing them in position again with thread.

### Righting of Specimens with One or More Arms Removed.

The removal of one or more arms gives opportunity to test and observe the correlation in the remaining arms more closely. This study was not carried far, and a single example will suffice.

*Experiment 3.*—A starfish was tested a number of times and it was found that it turned regularly on arms $e + a$. Arms $b$ and $d$ were now severed completely at the base. If the animal now continued to turn on $e$ and $a$, the only other arm concerned would be $c$, and it was expected that this would each time release as soon as coördination was established, allowing $e$ and $a$ to pull it over. It was proposed then to sever the nerve of $c$ in order to destroy its coördination with the other arms, and to see if they combined would still be able to pull it over, or whether coördination might be reëstablished through the mechanical pull, without nervous connection. In the first two trials after the operation the specimen turned on $e$ and $a$ as expected (Fig. 2), but in the third trial $c$ failed to coördinate properly and finally it with $a$ pulled over $e$, which was doubled under until these arms had crawled far enough to enable it to straighten out.

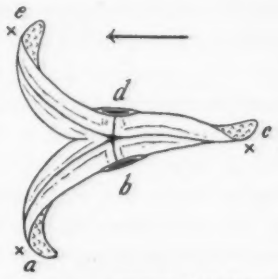



Fig. 2. Diagram of righting of a starfish with arms $b$ and $d$ removed. Nerves of other arms intact.

This experiment is not mentioned so much for the result obtained as to illustrate a possible method of studying coördination, impulses, and the relative use of the different arms in the starfish. It is important that the behavior of each specimen should be studied carefully in the normal condition before the operation is made.

### Behavior of Completely Severed Arms.

Romanes ('85, p. 294) found that "single rays detached from the organism crawl as fast and in as determinate a direction as do the entire animals," and that "when inverted, separated rays right themselves as quickly as do the unmutilated organisms."

The question naturally arises, however, in the light of Jennings' observations that there is commonly a preference for the use of certain rays in righting, whether these same rays will be the first to turn if all are detached and inverted, and whether they will twist in the same direction as when helping to right the entire starfish. Will subsequent locomotion also be in the direction with respect to each of the arms that it would have been if the arms were attached?

The detached arms have two principal methods of righting themselves when inverted. The first is to curl up orally until they topple over one way or the other, when the tube feet attach and quickly right the arm. The other method is for the tip of the arm to twist until the tube feet can attach. They then start to crawl and the remainder of the arm is soon pulled over much after the manner in which a flatworm rights itself (Pearl, '03, p. 673). A sufficient series of experiments was not performed to answer the questions propounded above, the results so far as obtained being somewhat contradictory. More extended experiments, however, would probably lead to more definite results.

## Conclusions.

As stated, the experiments mentioned above were rather incidental to other experiments on the starfish already reported (Cole, '13) and preliminary to others which it was hoped to continue. They are reported now not so much for the value of the results obtained as in the hope that they may stimulate further work along the same line. They do, however, seem to demonstrate, in so far as they go, the failure of the establishment of coördination or "unified impulses" in the starfish by direct pull of one part upon another when the nervous connection between these parts has been severed.

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# A NOTE ON THE SPERMATOGENESIS OF TENEBRIO MOLITOR.

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This study was suggested to me by Dr. Nettie M. Stevens, to whom I am deeply indebted for direction and help during the earlier part of the work, carried on while fellow in biology at Bryn Mawr College in 1912.[1]

The form studied is the common meal-worm, *Tenebrio molitor*. The worms were kept in the laboratory in a glass jar, and the testes dissected out as needed in the progress of the work. Five methods of fixation were used: Flemming's strong solution, Hermann's platino-acetic-osmic preparation, Gilson's mercuro-nitrate, and Bouin's fluid. After Flemming or Hermann fixation the testes were stained with iron-hæmatoxylin, and with safranin followed by gentian-violet or lichtgrün; after Bouin, with iron-hæmatoxylin and with thionin; after mercuro-nitrate, with Auerbach's fuchsin-green, with thionin, and with iron-hæmatoxylin.

The safranin and lichtgrün staining after either Flemming or Hermann fixation gave the most satisfactory results. By this method the structures of the nucleus were more clearly differentiated than by any of the other staining methods. The iron-hæmatoxylin and the thionin after any of the fixing agents were next in favor, but they were both useless for some of the most important stages, as they stained linin and chromatin alike. Two of the iron-hæmatoxylin slides, however, were destained sufficiently to give a faint differentiation in that respect. Auerbach's stain was a complete failure with my material; and although Miss Stevens and I both tried the Benda method repeatedly, in no instance did we obtain a typical stain. In two slides of Miss Stevens's the differentiation of the mitochondria

[1] Owing to Miss Stevens's sudden illness and death the work has had to be completed without her supervision. It is only through the great kindness of Dr. T. H. Morgan and Dr. E. B. Wilson, of Columbia University, that I have been enabled to prepare it for publication. My many thanks are due them both for their help and direction.

was fair, though by no means typical, the mitochondria being stained violet, the chromosomes a deep purple.

In 1905 Miss Stevens investigated the spermatogenesis of *Tenebrio molitor* (05*a*), paying special attention to the *X* chromosome. Her only reference to the conjugation stage is one short sentence: "A brief 'synapsis' or condensation stage occurs at the close of the last spermatogonial mitosis." She gives one figure of this stage and one of a slightly later stage, both drawn from iron-hæmatoxylin material. The drawing of the condensation stage shows a dense mass of chromatin at one side of the cell. The other figure shows what I have called the "large-loop" stage.

These figures and the accompanying descriptions indicate that Miss Stevens at that time supposed the conjugation to be of the type described by her for three of the Coleoptera and two of the Lepidoptera ('06*a*) and for *Diabrotica vittata*, another of the Coleoptera ('08). The type as described for these forms was telosynaptic. The very short loops that appear in the synizesis stage, "a prolongation of the last spermatogonial telophase," later straighten out and unite end to end to form loops which at first very often have knobs or slight irregularities at the point where the two chromosomes have come together. These loops then pass directly over into the diffuse spireme.

The appearances in my material indicate a considerable divergence from this type.

In the spermatogonia of the meal-worm there are nineteen large chromosomes and one small chromosome, making twenty in all, as shown in metaphase in Fig. 1. Figs. 2, *a* and *b*, show two views of a spermatogonial telophase from a cyst surrounded on three sides by spermatocytes. I have inclined to believe this stage the last telophase preceding the maturation divisions.

The next succeeding stage that I was able to find is shown in Fig. 3; this cell came from a cyst in which every cell except the one figured showed a dense closely massed clump of chromatin at one side of the cell. This appearance is so usual and so well known I did not think it necessary to show a figure of it. It is very similar to the stages called variously "synizesis" and "condensation," and as in Miss Stevens's material, seems to follow directly on the last spermatogonial telophase. There may be

between these two stages such intermediate conditions as Dr. Wilson found in *Lygæus* and *Oncopeltus* ('12). The presence of such stages would probably change the interpretation I have put upon the stages described. I was unable, however, to find any such intermediate conditions, and so have been forced to hold to the present interpretation.

These contraction stages were very common in my material, but the section figured, which I have interpreted as a cross section of such a clump, is a rather rare occurrence. This is to be expected, since sections through many different planes would give a side view of a mass crowded at one side of a cell, while a section through one plane only would give such a polar view as is shown in Fig. 3.

I have interpreted this contraction phase as the stage at which conjugation takes place, thus placing synapsis much earlier in the process of spermatogenesis than Miss Stevens had done in her earlier work. According to this interpretation, in Fig. 3, chromosomes *a* and *b*, *c* and *d*, *e* and *f*, *g* and *h*, *x* and *y*, are cross sections of pairs of chromosomes not yet united; chromosomes *j* and *k*, *l* and *m*, *o* and *p*, are side views of such pairs; and *q* and *r* are the tops of the thickened loops formed by two joined chromosomes.

Miss Stevens followed the work up to this point, and it was under her direction that this stage came to be interpreted as the conjugation stage. It was her opinion that the type here was telosynaptic.

As the side view of this stage shows only a very dense, irregular chromatin mass, and the cross sections of this stage are so rare, the actual meeting of the two chromosomes would be very difficult to see; I did not succeed in finding it. But the fact that at the end of this stage never more than ten chromosomes can be found in a cell, while just before it (that is, in the last spermatogonial telophase) twenty were present, is in itself proof that conjugation takes place at this time. And the appearance of the short loops (Figs. 4, 5, 6, 7, 8) as the condensation stage opens up, is an indication of the manner in which synapsis has occurred.

The next stage in the observed process is the transformation of these short thick loops into long loops, partly linin, partly chro-

matin. This, I believe, has not been hitherto described for any form and is the phenomenon so effectively brought out by the safranin-licht-grün process of staining, the linin staining a clear green, the chromatin a deep rich red. By no other staining method that I used was this differentiation clearly brought out. In almost all preparations these loops appear to be homogeneous, as Miss Stevens has drawn them in her paper ('05*a*). Figs. 5, 6, 7, and 8 show several stages in this process of loop-formation. How this transformation is brought about I did not determine. At this stage there is usually some amount of green-staining material mixed in with the chromatin mass. Whether the linin of the loops comes from the chromatin or from this diffuse green-staining material I did not determine.

There are always ten or fewer chromosomes in sections of cells at this stage, which is the period of general growth. The chromatin at the tops of the loops thickens and becomes dumbbell-shaped, and both the cell and the nucleus nearly double in size (compare Figs. 8 and 13).

Toward the end of this period of general growth, the loops begin to straighten out and then to become attached to each other by their linin ends, thus forming the spireme (Figs. 9, 10 and 12). The spireme never becomes diffuse, but retains this appearance of dense chromatin masses, like dumbbells, strung upon a linin thread. This thread gradually becomes fainter and more slender, until at the stage just preceding the first spermatocyte division, it is seen as an almost indistinguishable double line connecting the chromosomes (Fig. 13). Rarely during these later stages can a chromosome be found in a tetrad form (Fig. 12).

Fig. 14 shows these ten bivalent chromosomes in the prophase of a first maturation division, which is the reductional division.

## Conclusions.

1. No stages were found intervening between the last spermatogonial telophase and the contraction stage.

2. When the chromosomes have fully emerged from the contraction stage, they are in the form of loops, and are in the reduced number.

3. The loops formed by these bivalent chromosomes are not homogeneous, but are partly linin.

4. No diffuse "resting stage" was found, the chromatin being in the form of compact, definite bodies throughout the whole observed process.

5. No diffuse spireme was found.

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## Explanation of Plate I.

Fig. 1. Flemming; safranin and licht-grün. Spermatogonial metaphase, 20 chromosomes.

Figs. 2*a* and *b*. Ibid. Two views of a spermatogonial telophase.

Fig. 3. Flemming; safranin and licht-grün. Cross section of contraction stage; *a* and *b*, *c* and *d*, *e* and *f*, *g* and *h*, *x* and *y*, pairs of chromosomes in cross section, not yet united; *j* and *k*, *l* and *m*, *o* and *p*, pairs of chromosomes in side view, ready to unite; *q* and *r*, tops of short loops formed by the thickening, shortening and thickening of two united chromosomes.

Fig. 4. Flemming, safranin and licht-grün. A little later stage in the contraction phase, showing loops.

Figs. 5, 6, 7, 8. Flemming, safranin and licht-grün. Formation of large loops.

Fig. 9. Flemming, iron-hæmatoxylin. Large loops beginning to straighten out.

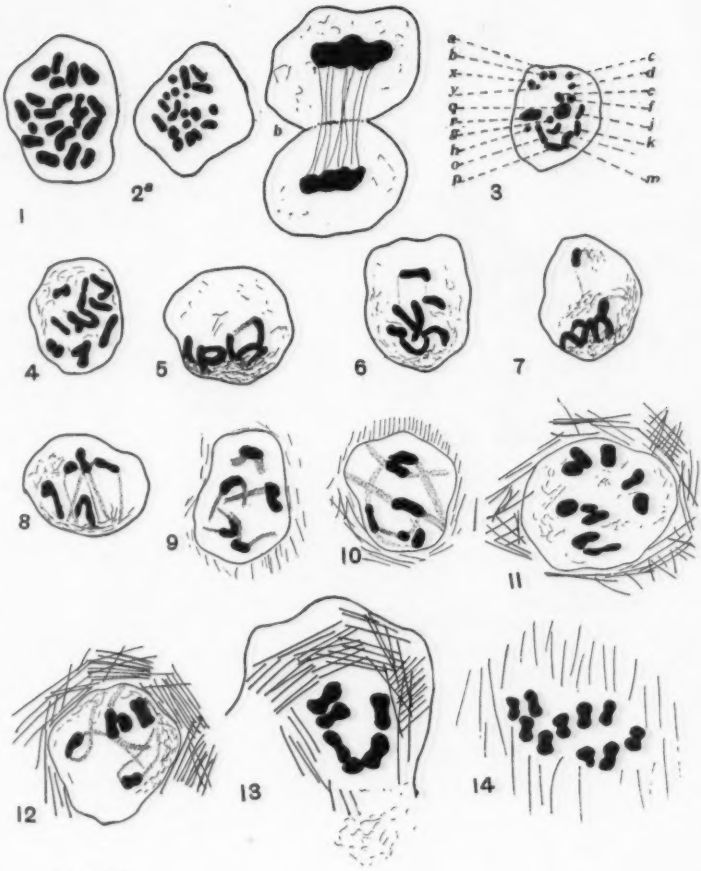
Fig. 10. Hermann, safranin and licht-grün. Formation of spireme.

Fig. 11. Hermann, safranin and licht-grün. Showing the ten chromosomes of this stage.

Fig. 12. Hermann, safranin and licht-grün. Spireme, showing tetrad.

Fig. 13. Flemming, safranin and licht-grün. Late spireme.

Fig. 14. Hermann, safranin and licht-grün. Prophase of first spermatocyte division, showing ten bivalent chromosomes, including the unequal pair.

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